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declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and
2. That the attached document is a true and correct translation of a certified copy of the following application, which was made by me to the best of my knowledge and belief.

- (a) Japanese Patent Application No. 2002-284263
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【Proof】 Necessary



[Document Name] Specification

[Title of the Invention] ADIP PROTEIN AND USE THEREOF

5 [Claims]

[Claim 1] A polynucleotide selected from any one of:

(a) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 2 or 4;

10 (b) a polynucleotide comprising a coding region of the nucleotide sequence of SEQ ID NO: 1 or 3;

(c) a polynucleotide comprising a nucleotide sequence encoding a protein having binding activity to afadin or actinin and comprising an amino acid sequence with a substitution, deletion, insertion and/or addition of one or more amino acids
15 in the amino acid sequence of SEQ ID NO: 2 or 4,; and

(d) a polynucleotide which hybridizes under stringent conditions with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3 and which encodes a protein having binding
20 activity to afadin or actinin.

[Claim 2] A polypeptide encoded by the polynucleotide of claim 1.

[Claim 3] A vector into which the polynucleotide of claim 1 is inserted.

25 [Claim 4] A host cell carrying the polynucleotide of claim 1 or the vector of claim 3.

[Claim 5] A method for producing the polypeptide of claim 2, comprising the steps of culturing the host cell of claim 4 and recovering the produced polypeptide from said host cell or
30 culture supernatant thereof.

[Claim 6] A polynucleotide which specifically hybridizes to the polynucleotide of claim 1 and which comprises at least 15 nucleotides.

35 [Claim 7] An antisense polynucleotide to the polynucleotide of claim 1 or to a portion thereof.

[Claim 8] An antibody which binds to the polypeptide of claim 2.

[Claim 9] A method of screening for a candidate compound of an actin cytoskeleton-controlling agent, comprising the steps of:

(a) contacting afadin or actinin with the polypeptide of claim 2 and a test compound;

5 (b) measuring the binding activity of afadin or actinin to the polypeptide of claim 2; and

(c) selecting the test compound which alters the binding activity, compared with that which occurs in the absence of the test compound.

10 [Claim 10] A method for assaying a heart disease which comprises the step of detecting the expression level of a gene encoding the polypeptide of claim 2 in a test subject.

[Claim 11] The method for assaying of claim 10, comprising the steps of:

15 (a) preparing an RNA sample from cardiac muscle cells of a test subject;

(b) measuring the amount of RNA encoding the polypeptide of claim 2 comprised in said RNA sample; and

(c) comparing the amount of the measured RNA with a control.

20 [Claim 12] The method for assaying of claim 10, comprising the steps of:

(a) preparing a protein sample from cardiac muscle cells of a subject;

25 (b) measuring the amount of the polypeptide of claim 2 comprised in said protein sample; and

(c) comparing the amount of the measured polypeptide with control.

[Claim 13] The disease of any one of claims 10 to 12, wherein the heart disease is myocardial infarction or myocarditis.

30

[Detailed Description of the Invention]

[0001] [Technical Field of Industrial Application]

The present invention relates to a novel afadin DIL domain-binding protein (ADIP) and a gene encoding the protein,
35 as well as methods of screening for pharmaceutical agents for diseases using the gene and diagnostic methods for the diseases.

[0002] [Prior Art]

Cells in multicellular organisms recognize neighboring cells, adhere to them and form intercellular junctions. Such junctions play essential roles in various cellular functions, including morphogenesis, differentiation, proliferation and migration (see Non-Patent Documents 1 to 6). In polarized epithelial cells, intercellular adhesion is mediated through a junctional complex composed of tight junctions (TJs), adherens junctions (AJs) and desmosomes. The junctional structures are typically aligned from the apical to basal sides, while desmosomes are independently distributed in other areas.

[0003] According to ultrastructural analysis, AJs were originally defined as closely apposed plasma membrane domains fortified with dense cytoplasmic plaques, lined with actin filament (F-actin) bundles (see Non-Patent Document 7). Molecular analysis showed that AJs are cell-cell adhesion sites assembled with actin-based cytoskeleton and several cytoplasmic components wherein typical cadherins function as cell adhesion molecules (see Non-Patent Documents 8 and 9). Similar to other typical cadherins, E-cadherin is a single-pass transmembrane protein whose extracellular domain mediates homophilic recognition and adherens junction in a Ca^{2+} -dependent manner (see Non-Patent Document 10). E-cadherin associates with actin cytoskeleton through peripheral membrane proteins, including α -catenins, β -catenins, γ -catenins, α -actinin and vinculin (see Non-Patent Documents 11 to 14). β -Catenin directly interacts with the cytoplasmic tail of E-cadherin and connects E-cadherin to α -catenin that directly binds to F-actin (see Non-Patent Document 15). α -Actinin and vinculin are F-actin-binding proteins that directly bind to α -catenin (see Non-Patent Documents 13, 14, and 16).

[0004] The association of E-cadherin with the actin cytoskeleton through these peripheral membrane proteins potentiate cell-cell adhesion by E-cadherin (see Non-Patent Documents 1 and 17).

[0005] The present inventors discovered that another cell-cell adhesion molecule (nectin) and F-actin-binding protein (afadin), which associates with nectin, also localize at AJs (see Non-Patent Documents 18 and 19). Nectin and afadin strictly localized at AJs and, using ultrastructural analysis, they were defined as closely apposed plasma membrane domains fortified with dense cytoplasmic plaques lined with F-actin bundles (see Non-Patent Documents 7, 18, and 19). In contrast, E-cadherin is concentrated at AJs but is more widely distributed from the apical to basal sides of the lateral plasma membranes (see Non-Patent Documents 2 and 20). Nectin is a Ca^{2+} -independent immunoglobulin-like cell-cell adhesion molecule (see Non-Patent Documents 19 and 21 to 25). At present, nectin comprises a family consisting of four members, i.e., nectin-1, -2, -3 and -4. All nectins, with the exception of nectin-4, have two or three splice variants, i.e., nectin-1 α , -1 β , -2 α , -2 δ , -3 α , -3 β and -3 γ isoforms (see Non-Patent Documents 19 and 24 to 30). Nectin-1 was originally identified as one of the poliovirus receptor-related proteins (PRR1) (see Non-Patent Document 29). Nectin-2 was originally identified as a murine homolog of human poliovirus receptor protein (see Non-Patent Document 26), but later turned out to be another poliovirus receptor-related protein (PRR2) (see Non-Patent Documents 28 and 29). PRR1 and PRR2 were later shown to serve as receptors for α -herpesvirus, facilitating their invasion and intercellular spreading, and thus were renamed HveC and HveB, respectively (see Non-Patent Documents 30 to 35). All members of nectin have an extracellular domain with three immunoglobulin-like loops, a single transmembrane region and a cytoplasmic region. Furthermore, all of them, with the exception of nectin-1 β , nectin-3 γ and nectin-4, have a conserved motif of four amino acid residues (Glu/Ala-X-Tyr-Val) at their carboxy terminus, and this motif binds to the PDZ domain of afadin (see Non-Patent Documents 18, 19, 24, and 25).

[0006] Afadin has at least two splice variants, namely, l- and s-afadins (see Non-Patent Document 18). l-Afadin, the

larger splice variant, binds to nectin and, through its F-actin binding domain, to F-actin. l-Afadin binds to the side of F-actin but does not crosslink with it to form bundles. l-Afadin has two Ras-associated domains (RA), a forkhead-associated domain (FHA), a dilute (DIL) domain, a PDZ domain, two proline rich domains (PR) and an F-actin-binding PR domain (see Fig. 1A). The DIL domain is found in afadin, dilute (DIL) and V-type myosin, comprising Myo2 and Myo4. However, the function of this domain is still unknown (see Non-Patent Document 36). Recent findings that the Myo4 region containing the DIL domain binds to an adaptor protein, She3 (see Non-Patent Documents 37 and 38) indicates that the DIL domain is involved in protein-protein interactions.

[0007] s-Afadin, the smaller splice variant, has two RA, FHA, DIL, PDZ and two PR domains, but lacks the F-actin-binding PR domain. Human s-afadin is identical to the gene product of AF-6, a gene that has been identified as an ALL-1 fusion partner involved in acute myeloid leukemias (see Non-Patent Document 39). Unless otherwise specified, "afadin" refers to l-afadin in the present specification.

[0008] Nectin supplies E-cadherin- β -catenin complex to the nectin-based cell-cell adhesion sites through afadin and α -catenin in fibroblast and epithelial cells (see Non-Patent Documents 40 and 41). Furthermore, nectin supplies TJ components, including ZO-1, claudin, occludin and junction adhesion molecule (JAM), to the nectin-based cell-cell adhesion sites through afadin in fibroblasts and epithelial cells (see Non-Patent Documents 41 to 43). Claudin is an important cell-cell adhesion molecule that forms TJ strands (see Non-Patent Documents 44 to 47), and occludin and JAM are other transmembrane proteins at TJs (see Non-Patent Documents 46 to 48). Claudin, occludin and JAM interact with an F-actin-binding scaffold molecule, ZO-1 (see Non-Patent Documents 49 to 60). In epithelial cells of afadin (-/-) mice and (-/-) embryoid bodies, the proper organization of AJs and TJs is impaired (see Non-Patent Document 61). By positional cloning,

nectin-1 has recently been related to cleft lip/palate-ectodermal dysplasia, which is characterized by cleft lip/palate, syndactyly, mental retardation and ectodermal dysplasia (see Non-Patent Document 62).

5 [0009] In addition, the present inventors have recently identified that the nectin-afadin system is involved in the formation of synapses of neurons in cooperation with N-cadherin (see Non-Patent Document 63) and that the nectin-afadin system constitutes an important adhesion system in the organization of
10 Sertoli cell-spermatid junction of the testis (see Non-Patent Document 64). Therefore, nectin and afadin are important for the organization of a wide variety of intercellular junctions either together with or independently of known cell adhesion molecules. However, the molecular mechanism as to how the
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[0011] [Problems to be Solved by the Invention]

- 20 This need in the art led to the present invention, and the objective of the present invention is to provide a novel afadin DIL domain-binding protein (ADIP) gene. Another objective of the present invention is to provide uses for this identified novel ADIP.

[0012] [Means to Solve the Problems]

- 25 The present inventors conducted dedicated research to solve the above-mentioned problems. First, the present inventors identified afadin-binding proteins by yeast two-hybrid screening to analyze how the nectin-afadin system organizes AJs and TJs. As a result, the present inventors
30 succeeded in identifying a novel afadin-binding protein, ADIP (afadin DIL domain-interacting protein), that binds to the DIL domain of afadin which was used as a bait.

- [0013] ADIP was further revealed to bind to α -actinin, which is an F-actin-bundling protein known to indirectly
35 associate with E-cadherin through its direct binding to α -catenin (K.A. Knudsen, A.P. Soler, K.R. Johnson and M.J.

Wheelock, "Interaction of alpha-actinin with the cadherin/catenin cell-cell adhesion complex via alpha-catenin." J. Cell Biol. 1995, 130: 67-77). These results suggested that ADIP may be associated with the nectin-afadin and E-cadherin-catenin systems through α -actinin, and that ADIP may be involved in the organization of the actin cytoskeleton at AJs through afadin and α -actinin. Therefore, ADIP is expected to be useful for the evaluation of agents that control the actin cytoskeleton.

[0014] Moreover, ADIP may be useful as a functional marker of the intercalate disc for heart diseases, such as myocardial infarction and myocarditis, due to its extremely high expression at the intercalate disc of cardiac muscle cells.

[0015] Specifically, the present invention relates to a novel ADIP gene and use thereof. More specifically, the present invention provides:

[1] a polynucleotide selected from any one of:

(a) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 2 or 4;

(b) a polynucleotide comprising a coding region of the nucleotide sequence of SEQ ID NO: 1 or 3;

(c) a polynucleotide comprising a nucleotide sequence encoding a protein having binding activity to afadin or actinin and comprising an amino acid sequence with a substitution, deletion, insertion and/or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 2 or 4,; and

(d) a polynucleotide which hybridizes under stringent conditions with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3 and which encodes a protein having binding activity to afadin or actinin;

[2] a polypeptide encoded by the polynucleotide of [1];

[3] a vector into which the polynucleotide of [1] is inserted;

[4] a host cell carrying the polynucleotide of [1] or the vector of [3];

[5] a method for producing the polypeptide of [2], comprising the steps of culturing the host cell of [4] and recovering the

produced polypeptide from said host cell or culture supernatant thereof;

[6] a polynucleotide which specifically hybridizes to the polynucleotide of [1] and which comprises at least 15 nucleotides;

[7] an antisense polynucleotide to the polynucleotide of [1] or to a portion thereof;

[8] an antibody which binds to the polypeptide of [2];

[9] a method of screening for a candidate compound of an actin cytoskeleton-controlling agent, comprising the steps of:

(a) contacting afadin or actinin with the polypeptide of [2] and a test compound;

(b) measuring the binding activity of afadin or actinin to the polypeptide of [2]; and

(c) selecting the test compound which alters the binding activity, compared with that which occurs in the absence of the test compound;

[10] a method for assaying a heart disease which comprises the step of detecting the expression level of a gene encoding the polypeptide of [2] in a test subject;

[11] the method for assaying of [10], comprising the steps of:

(a) preparing an RNA sample from cardiac muscle cells of a test subject;

(b) measuring the amount of RNA encoding the polypeptide of [2] comprised in said RNA sample; and

(c) comparing the amount of the measured RNA with a control;

[12] the method for assaying of [10], comprising the steps of:

(a) preparing a protein sample from cardiac muscle cells of a subject;

(b) measuring the amount of the polypeptide of [2] comprised in said protein sample; and

(c) comparing the amount of the measured polypeptide with control; and

[13] the disease of any one of [10] to [12], wherein the heart disease is myocardial infarction or myocarditis.

[0016] Mode for Carrying Out the Invention

The present invention provides polynucleotides encoding a novel protein, ADIP, which binds to the afadin DIL domain. The polynucleotide sequence encoding mouse ADIP and the amino acid
5 sequence of polypeptide encoded by the polynucleotide, identified by the present inventors and encompassed by the present invention, are shown in SEQ ID NOs: 1 and 2, respectively. Moreover, the polynucleotide sequence encoding rat ADIP and the amino acid sequence of polypeptide encoded by
10 the polynucleotide, also identified by the present inventors and encompassed by the present invention, are shown in SEQ ID NOs: 3 and 4, respectively.

[0017] The present invention also relates to polypeptides functionally equivalent to the polypeptides identified by the
15 present inventors and polynucleotides encoding such functionally equivalent polypeptides. Herein, the phrase "functionally equivalent" means that the subject polypeptide has equivalent biological characteristics to a polypeptide identified by the present inventors. An example of a
20 biological characteristic of ADIP includes the binding activity to afadin and/or actinin. Therefore, the present invention includes polynucleotides comprising the nucleotide sequence encoding a protein that consists of an amino acid sequence with a substitution, deletion, insertion and/or addition of one or
25 more amino acids in the amino acid sequence of SEQ ID NO: 2 or 4, and that retains the binding activity to afadin or actinin. Moreover, the present invention also includes polynucleotides that hybridize under stringent conditions with a DNA consisting of the nucleotide sequences of SEQ ID NO: 1 or 3, and that
30 encode proteins having the binding activity to afadin or actinin. The measurement of the above-mentioned "binding activity" can be conducted by methods well known to those skilled in the art, such as the yeast two-hybrid system. The term "actinin" herein generally indicates " α -actinin".

35 [0018] The polynucleotides of the present invention can be isolated by methods well known to those skilled in the art.

Examples of such methods include hybridization technique (E.M. Southern, J. Mol. Biol. 1975, 98: 503-517) and polymerase chain reaction (PCR) technique (R.K. Saiki et al., Science 1985, 230: 1350-1354; R.K. Saiki et al., Science 1988, 239: 487-491).

5 More specifically, those skilled in the art can generally isolate polynucleotides highly homologous to the polynucleotides consisting of the nucleotide sequence of SEQ ID NO: 1 or 3 from other animals (such as human), using the polynucleotide consisting of the nucleotide sequence of SEQ ID
10 NO: 1 or 3 or parts thereof as probes or using the oligonucleotide which specifically hybridizes with the polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 1 or 3 as primers. Furthermore, polynucleotides that can be isolated by hybridization techniques or PCR techniques and
15 that hybridize with polynucleotides consisting of the nucleotide sequence of SEQ ID NO: 1 or 3 are also included in the polynucleotides of the present invention. Examples of such polynucleotides include polynucleotides encoding the human homolog of ADIP.

20 [0019] Hybridization reactions to isolate polynucleotides as described above are preferably conducted under stringent conditions. In the present invention, stringent hybridization conditions indicate conditions comprising: 6 M urea, 0.4% SDS and 0.5x SSC, and those having a stringency equivalent to these
25 conditions. Polynucleotides with higher homology are expected to be isolated under conditions with higher stringency, for example, 6 M urea, 0.4% SDS and 0.1x SSC. The polynucleotides thus isolated are expected to have a high homology at the amino acid level to the amino acid sequence of SEQ ID NO: 2 or 4.
30 Herein, "high homology" means a sequence identity of at least 70% or more, more preferably 80% or more, further more preferably 90% or more, and most preferably 95% or more, in the whole amino acid sequence.

[0020] The identity in the amino acid sequences and
35 nucleotide sequences can be determined using the BLAST algorithm by Karlin and Altschul (S. Karlin and S.F. Altschul,

Proc. Natl. Acad. Sci. USA. 1990, 87: 2264-2268; S. Karlin and S.F. Altschul, Proc. Natl. Acad. Sci. USA. 1993, 90: 5873-5877). The BLAST algorithm-based programs, called BLASTN and BLASTX, have been developed (S.F. Altschul et al., J. Mol. Biol. 1990, 215: 403). When a nucleotide sequence is analyzed according to BLASTN, parameters are set, for example, at score= 100 and word length= 12. On the other hand, parameters used for the analysis of amino acid sequences by BLASTX are set, for example, at score= 50 and word length= 3. Default parameters of each program are used when BLAST and Gapped BLAST programs are used. Specific procedures for such analysis are known (<http://www.ncbi.nlm.nih.gov>).

[0021] Polynucleotides of the present invention include genomic DNAs, cDNAs, and chemically synthesized DNAs. A genomic DNA or cDNA can be prepared according to conventional methods known to those skilled in the art. For example, genomic DNA can be prepared as follows: (i) extracting genomic DNA from organisms comprising the polynucleotide encoding ADIP; (ii) constructing a genomic library (using, for example, a plasmid, phage, cosmid, BAC or PAC, as a vector); (iii) spreading the library; and then (iv) conducting colony hybridization or plaque hybridization using probes prepared based on the polynucleotide (e.g., SEQ ID NO: 1 or 3) encoding an ADIP of the present invention. Alternatively, the genomic DNA can be prepared by PCR, using primers specific to a polynucleotide (e.g., SEQ ID NO: 1 or 3) encoding the ADIP of the present invention. On the other hand, the cDNA can be prepared, for example, as follows: (i) synthesizing cDNAs based on mRNA extracted from organisms comprising the polynucleotide encoding an ADIP; (ii) constructing a cDNA library by inserting the synthesized cDNA into vectors, such as λ ZAP; (iii) spreading the cDNA library; and (iv) conducting colony hybridization or plaque hybridization as described above. Alternatively, the cDNA can also be prepared by PCR.

[0022] The present invention also provides polynucleotides encoding proteins structurally similar to the protein

consisting of the amino acid sequence of SEQ ID NO: 2 or 4. Such polynucleotides include polynucleotides consisting of a nucleotide sequence encoding a protein comprising an amino acid sequence with a substitution, deletion, insertion, and/or addition of one or more amino acids in these proteins. There is no limitation on the number and sites of the amino acid mutation in the above proteins so long their functions are retained. The number of mutations may be typically 10% or less, preferably 5% or less, and more preferably 1% or less of the total amino acids.

[0023] Exemplary methods well known to those skilled in the art for preparing an above-described polynucleotide include site-directed mutagenesis (W. Kramer and H-J. Fritz, Methods Enzymol. 1987, 154: 350) for introducing mutations in the polynucleotide, in addition to the hybridization and PCR techniques described above. The amino acid sequence of a protein may also be mutated in nature due to a mutation of the nucleotide sequence encoding the protein. Additionally, degeneracy mutant polynucleotides, in which nucleotide sequence mutations do not give rise to any amino acid sequence mutations in the protein (degeneracy mutants), are also included in the present invention. Furthermore, the present invention also includes the polypeptides which are encoded by the aforementioned polynucleotides of the present invention.

[0024] The present invention provides vectors containing polynucleotides of the present invention, host cells retaining polynucleotides or the vector of the present invention, and methods for producing polypeptides of the present invention utilizing the host cells.

[0025] The vector of the present invention is not limited so long as the DNA inserted in the vector is stably retained. For example, pBluescript vector (Stratagene) is preferable as a cloning vector when using *E. coli* as the host. When using a vector for producing a polypeptide of the present invention, an expression vector is particularly useful. The expression vector is not particularly limited, so long as it expresses

polypeptides *in vitro*, in *E. coli*, in cultured cells or *in vivo*. Preferable examples of the expression vectors include the pBEST vector (ProMega) for *in vitro* expression, the pET vector (Invitrogen) for the expression in *E. coli*, the pME18S-FL3
5 vector (GenBank Accession No. AB009864) for the expression in cultured cells and the pME18S vector (Mol. Cell Biol. 1988, 8: 466-472) for *in vivo* expression. The insertion of a DNA of the present invention into a vector can be carried out by
10 conventional methods, for example, the ligase reaction using restriction enzyme sites (Current Protocols in Molecular Biology, edit. Ausubel et al., Publish. John Wiley & Sons, 1987, Section 11.4-11.11).

[0026] The host cell to which the vector of the present invention is introduced is not specifically limited, and
15 various host cells can be used according to the objects of the present invention. Examples of cells for expressing the polypeptides include bacterial cells (e.g., *Streptococcus*, *Staphylococcus*, *E. coli*, *Streptomyces*, *Bacillus subtilis*), fungal cells (e.g., yeast, *Aspergillus*), insect cells (e.g.,
20 *Drosophila* S2, *Spodoptera* SF9), animal cells (e.g., CHO, COS, HeLa, C127, 3T3, BHK, HEK293, Bowes melanoma cell) and plant cells. The introduction of a vector to a host cell can be carried out by conventional methods, such as the calcium phosphate precipitation method, the electroporation method
25 (Current protocols in Molecular Biology, edit. Ausubel et al., Publish. John Wiley & Sons, 1987, Section 9.1-9.9), the Lipofectamine method (GIBCO-BRL), the microinjection method.

[0027] Appropriate secretion signals can be incorporated into the polypeptide of interest in order to secrete the
30 polypeptides expressed in a host cell into the lumen of endoplasmic reticulum, into cavity around the cell or into the extracellular environment. These signals may be endogenous signals or signals from a different species to the objective polypeptide.

35 [0028] When a polypeptide of the present invention is secreted into the culture media, the culture media is collected

to collect the polypeptide of the present invention. When a polypeptide of the present invention is produced intracellularly, the cells are first lysed, and then, the polypeptides are collected.

5 [0029] In order to collect and purify a polypeptide of the present invention from a recombinant cell culture, methods known in the art, comprising ammonium sulfate or ethanol precipitation, extraction by acid, anionic or cationic exchange chromatography, phosphocellulose chromatography, hydrophobic
10 interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography, can be used.

[0030] The present invention provides nucleotides, having a chain length of at least 15 nucleotides, which are
15 complementary to a polynucleotide identified by the present inventors (a polynucleotide or a complementary strand thereof comprising the nucleotide sequence of SEQ ID NO: 1 or 3). Herein, the phrase "complementary strand" indicates one strand of a double strand nucleic acid composed of A:T (A:U in case of
20 RNA) and G:C base pairs to the other strand. Furthermore, the term "complementary" is not only used for nucleotides that completely match within a continuous region of at least 15 sequential nucleotides, but also those having a homology of at least 70%, preferably at least 80%, more preferably 90% and
25 further more preferably 95% or higher within the nucleotide region. The homology may be determined using an algorithm described herein. Such nucleotides may be used as probe for detection and isolation of the polynucleotides of the present invention, or as primers for amplification of the nucleotides
30 of the present invention. Polynucleotides used as primers generally have a chain length of 15 to 100 nucleotides and preferably 15 to 35 nucleotides. Alternatively, polynucleotides used as probes may have a chain length of at least 15 nucleotides, preferably at least 30 nucleotides,
35 containing at least a portion or the whole sequence of a DNA of the present invention. Such polynucleotides preferably

hybridize specifically to a polynucleotide encoding a polypeptide of the present invention. The phrase "hybridize specifically" means that a nucleotide hybridizes under a normal hybridization condition, preferably under a stringent condition, with a polynucleotide (SEQ ID NO: 1 or 3) identified by the present inventors, but not with DNAs encoding other polypeptides.

[0031] Furthermore, the polynucleotides include those that suppress the expression of genes encoding the polypeptides of the present invention. Such polynucleotides include antisense polynucleotides (antisense DNA/RNA; antisense RNAs, which are complementary to transcriptional products of the genes encoding the polypeptides of the present invention, and DNAs encoding the RNAs) and ribozymes (DNAs encoding RNAs having ribozyme activities to specifically cleave transcriptional products of the genes encoding the polypeptides of the present invention).

[0032] There are a plurality of causes, such as those described below, for the effect of an antisense polynucleotide that suppresses the expression of a target gene: inhibition of transcription initiation by the formation of a triple strand; suppression of transcription through hybridization with a local open loop conformation site formed by an RNA polymerase; inhibition of transcription by hybridization with RNA, which is in course of synthesis; suppression of splicing through hybridization at a junction of intron and exon; suppression of splicing through hybridization with a spliceosome forming site; suppression of translocation from the nuclei to cytoplasm through hybridization with mRNAs; suppression of splicing through hybridization with capping sites or poly(A) addition sites; suppression of translation initiation through hybridization with a translation initiation factor binding site; suppression of translation through hybridization with the ribosome binding site near the initiation codon; inhibition of elongation of peptide chain through hybridization with the translation regions and polysome binding sites of mRNAs; and suppression of expression of genes by hybridization with the

interaction sites between nucleic acids and proteins. These actions inhibit the processes of transcription, splicing or translation to suppress the expression of a target gene (Hirajima and Inoue, "New Biochemistry Experimental Course No. 2, Nucleic Acid IV, Replication and Expression of Genes", Japan Biochemical Society ed., Tokyo Kagaku Doujin, 1993, 319-347).

[0033] The antisense polynucleotides used in the present invention may suppress the expression of the target gene through any of the above-mentioned actions. According to one embodiment, an antisense sequence designed to be complementary to a non-translated region near the 5' terminus of mRNA of a gene may effectively inhibit the translation of the gene. However, sequences which are complementary to the coding region or the 3' non-translated region can be also used. As described above, polynucleotides containing antisense sequences not only to the translated region of a gene, but also those to sequences of non-translated regions are included in the antisense polynucleotides used in the present invention. The antisense polynucleotides used in the present invention are linked downstream of an appropriate promoter and a sequence including a transcriptional termination signal is preferably linked to the 3'-side. The sequence of the antisense polynucleotide is preferably complementary to the target gene or a part thereof; however, so long as the expression of the gene can be effectively inhibited, it does not have to be completely complementary to the target sequence. The transcribed RNA is preferably 90% or more, most preferably 95% or more, complementary to the transcribed product of the target gene. In order to effectively inhibit the expression of the target gene using an antisense sequence, the antisense polynucleotide has at least a chain length of 15 nucleotides or more, preferably 100 nucleotides, more preferably 500 nucleotides or more, and usually has a chain length 3000 nucleotides or less, preferably 2000 nucleotides or less to cause an antisense effect.

[0034] The antisense polynucleotide can be prepared, for example, by the phosphorothionate method (Stein, "Physicochemical properties of phosphorothioate oligodeoxynucleotides." Nucleic Acids Res. 1988, 16: 3209-3221) based on the sequence information of a polynucleotide (for example, the sequence of SEQ ID NO: 1 or 3) encoding a polypeptide of the present invention.

[0035] Furthermore, suppression of the expression of endogenous genes can be also achieved utilizing polynucleotides encoding ribozymes. Ribozymes are RNA molecules having catalytic activity. There exist ribozymes with various activities, and research on ribozymes as an enzyme for truncating RNA allowed for the design of ribozymes that cleave RNAs in a site-specific manner. There are ribozymes with a size of 400 nucleotides or more, such as Group I intron type ribozymes and M1RNA, a member of RNaseP, as well as those which have an active domain of about 40 nucleotides, called hammer-head type or hairpin type ribozyme (Makoto Koizumi and Eiko Ohtsuka, Protein Nucleic Acid and Enzyme (PNE) 1990, 35: 2191).

[0036] For example, the self-cleavage domain of the hammer-head type ribozyme cleaves the 3'-side of C15 of G13U14C15. A base pair formation of the U14 with the A at position 9 is important for the activity and the cleavage is demonstrated to proceed even if the C at position 15 is A or U (M. Koizumi et al., FEBS Lett. 1988, 228: 225). Restriction enzymatic RNA-cleaving ribozymes recognizing sequences of UC, UU or UA in a target RNA may be generated by designing the substrate binding site of the ribozyme complementary with the RNA sequence near the target site (M. Koizumi et al., FEBS Lett. 1988, 239: 285; Makoto Koizumi and Eiko Ohtsuka, Protein Nucleic Acid and Enzyme (PNE) 1990, 35: 2191); M. Koizumi et al., Nucleic Acids Res. 1989, 17: 7059).

[0037] Furthermore, the hairpin type ribozymes are also useful for the objective of the present invention. The hairpin type ribozymes are found on, for example, the minus chain of a satellite RNA of tobacco ringspot virus (J.M. Buzayan, Nature

1986, 323: 349). It is also demonstrated that this ribozyme can also be designed to cause a target specific RNA cleavage (Y. Kikuchi and N. Sasaki, Nucleic Acids Res. 1992, 19: 6751; Y. Kikuchi, Chemistry and Organism 1992, 30: 112).

5 [0038] When a polynucleotide suppressing the expression of a gene encoding a polypeptide of the present invention is used in gene therapy, it may be administered to a patient by the ex vivo method, in vivo method and the like, using, for example, viral vectors such as retroviral vector, adenoviral vector and
10 adeno-associated viral vectors; and non-viral vectors such as liposome.

[0039] The present invention provides antibodies that bind to a polypeptide of the present invention. Herein, the term "antibodies" encompasses polyclonal antibodies, monoclonal
15 antibodies, chimeric antibodies, single-stranded antibodies, humanized antibodies and Fab fragments including Fab or other products of the immunoglobulin expression library.

[0040] A polypeptide of the present invention or fragment or analog thereof, or a cell that expresses these can be used
20 as an immunogen for producing antibodies binding to a polypeptide of the present invention. The antibodies are preferably immunospecific to a polypeptide of the present invention. The term "immunospecific" means that the antibody has substantially higher affinity to a polypeptide of the
25 present invention than to other polypeptides.

[0041] The antibodies binding to a polypeptide of the present invention can be prepared by conventional methods for those skilled in the art. For example, a polyclonal antibody can be obtained as follows: Small animals such as rabbit are
30 immunized with a polypeptide of the present invention or a fusion protein thereof with GST to obtain serum. The polyclonal antibody may be prepared by purifying the serum through ammonium sulfate precipitation, protein A or protein G column, DEAE ion exchange chromatography, or affinity column,
35 wherein the polypeptide of the present invention is coupled, and such. On the other hand, a monoclonal antibody can be

prepared as follows: A polypeptide of the present invention is administered to a small animal such as a mouse and the spleen is subsequently excised from the mouse and ground to separate cells. Then, the cells are fused with mouse myeloma cells using reagents such as polyethylene glycol, and clones that produce antibodies binding to the polypeptide of the present invention are selected from these fused cells (hybridoma). The obtained hybridoma is then transplanted into the peritoneal cavity of a mouse and ascites is collected from the mouse. The monoclonal antibodies can be prepared by purifying the obtained monoclonal antibodies using, for example, ammonium sulfate precipitation; protein A or protein G column; DEAE ion exchange chromatography; affinity column wherein the polypeptides of the present invention are coupled; and such.

[0042] The antibodies of the present invention can be used for the isolation, identification and purification of the polypeptides of the present invention and cells expressing them.

[0043] The polypeptides of the present invention can be used to screen for candidate compounds for actin cytoskeleton-controlling agents. Actin cytoskeleton-controlling agents may be used as therapeutic agents for diseases associated with abnormal expression (function) of a polypeptide of the present invention. Molecules to be identified may be naturally-occurring molecules as well as artificially synthesized structural or functional imitated molecules. The polypeptides of the present invention are believed to be involved in various biological functions, including many pathologies. Thus, the detection of compounds that activate the polypeptides of the present invention and compounds that inhibit the activation of the polypeptides of the present invention is desired.

[0044] The present invention provides methods of screening for candidate compounds for actin cytoskeleton-controlling agents.

[0045] According to the present invention, first, afadin or actinin is contacted with a candidate compound and a polypeptide of the present invention, and then, the binding

activity of afadin or actinin to the polypeptide of the present invention is measured. Next, the compound that modifies (*i.e.*, increases or suppresses) the above-mentioned binding activity, as compared with the binding activity without the test compound, is selected.

[0046] Compounds modifying the binding activity of a polypeptide of the present invention to afadin or actinin that are isolated by the above-mentioned screening method of the present invention are expected to serve as therapeutic agents, for example, for heart diseases such as myocardiac infarction and myocarditis. Moreover, the compounds can be further subjected to the above-mentioned screening method of the present invention as test compounds.

[0047] The binding activity as described above can be appropriately measured by methods well known in the art, such as the yeast two-hybrid system. There is no particular limitation on the test compounds; for example, various known compounds and peptides (for example, those registered in the Chemical File); and random peptide groups that are produced by applying the phage-display method (*J. Mol. Biol.* 1991, 222: 301-310) may be utilized. Furthermore, culture supernatants of microorganisms, natural components derived from plants and marine organisms and such can be used as the object of the screening. Other examples include extracts from biotic tissues, extracted solutions from cells, expression products of gene libraries and such, though the invention is not limited thereto.

[0048] The present invention also relates to methods for examining diseases related to abnormal expression of the genes encoding the polypeptides of the present invention. The polypeptides of the present invention are considered to have important functions *in vivo*, and thus, abnormal expression thereof may cause various diseases. Therefore, assay of diseases may be accomplished using expression of the polypeptides of the present invention as an index.

[0049] The phrase "assay of diseases" includes not only tests to draft therapeutic strategy for a subject who exhibits

the symptom of a disease, but also tests for preventing diseases by determining whether the subject is susceptible to the disease or tests for determining whether the subject is already affected to the disease or not.

5 [0050] Since ADIPs are strongly expressed at the intercalated disc of cardiac muscle cells, abnormal expression of ADIP may cause heart diseases. Therefore, examples of a disease of the present invention typically include heart diseases, more specifically, myocardial infarction and
10 myocarditis.

 [0051] One embodiment of the test methods of the present invention is a method comprising the step of detecting, in a subject, mutation of a gene encoding a polypeptide of the present invention or in the expression control regions thereof.

15 [0052] One of the methods is a method in which an examination is accomplished by directly determining the nucleotide sequence of a gene encoding a polypeptide of the present invention or its expression control region in a subject. According to this method, first, a DNA sample is prepared from
20 a subject. The DNA sample can be prepared from chromosomal DNA or RNA extracted from cells of the subject, for example, cardiac muscle cells. In order to prepare a DNA sample for the present method from a chromosomal DNA, a genomic library may be produced by, for example, digesting the chromosomal DNA with
25 appropriate restriction enzymes, and then cloning the digested DNA into a vector. On the other hand, for example, a cDNA library may be prepared from RNA using reverse transcriptase to prepare a DNA sample for the present method from RNA. Next, DNA containing a gene encoding a polypeptide of the present
30 invention or the expression control region thereof is isolated according to the present method. The isolation of the DNA can be carried out by screening the genomic library or cDNA library, using probes hybridizing with the DNA containing the gene encoding the polypeptide of the present invention or its
35 expression control region. The isolation of the DNA can also be carried out by PCR using the genomic DNA library, cDNA

library or RNA as the template, and primers hybridizing to a DNA containing a gene encoding a polypeptide of the present invention or its expression control region. Then, the nucleotide sequence of the isolated DNA is determined according to the present method. The determination of the nucleotide sequence of selected DNAs can be carried out by methods known to those skilled in the art. According to the present method, the determined nucleotide sequence of the DNA is then compared with a control. The "control" as used herein refers to a nucleotide sequence of DNAs containing a gene encoding a normal (wild type) polypeptide of the present invention or its expression control region. When the nucleotide sequence of the DNA of a subject differs from that of the control as a result of the comparison above, the subject is judged to be affected with disease or is in danger of developing the disease.

[0053] According to the test method of the present invention, various methods can be used other than the method of directly determining the nucleotide sequence of a DNA derived from a subject as described above.

[0054] In one embodiment of the present method, first, a DNA sample is prepared from a subject and digested with restriction enzymes. Then, the DNA fragments are separated in accordance with their size, followed by comparison of the sizes of the detected DNA fragments with those of a control. Alternatively, in another embodiment, a DNA sample is first prepared from a subject. Then, DNA containing a gene encoding a polypeptide of the present invention or its expression control region is amplified, and the amplified DNAs are digested with restriction enzymes. After separating the DNA fragments according to their size, the sizes of the detected DNA fragments are compared with those of a control.

[0055] Such methods include a method utilizing the Restriction Fragment Length Polymorphism/RFLP and the PCR-RFLP method. Specifically, when variations exist for the recognition sites of a restriction enzyme, or when insertion(s) or deletion(s) of nucleotide(s) exists in a DNA fragment

generated by a restriction enzyme treatment, the sizes of fragments that are generated after the restriction enzyme treatment vary in comparison with those of a control. By amplifying the portion containing the mutation by PCR and then
5 treating with respective restriction enzymes, these mutations can be detected as a difference in the mobility of bands after electrophoresis. Alternatively, the presence or absence of mutations can be detected by carrying out Southern blotting with a probe DNA of the present invention after treating the
10 chromosomal DNA with these restriction enzymes followed by electrophoresis. The restriction enzymes to be used can be appropriately selected in accordance with respective mutations. According to this method, the Southern blotting can be conducted not only on the genomic DNA but also on cDNAs
15 directly digested with restriction enzymes, wherein the cDNAs are converted by the use of a reverse transcriptase from RNAs prepared from subjects. Alternatively, the difference in mobility can also be examined after amplifying DNAs containing a gene encoding a polypeptide of the present invention or its
20 expression control region by PCR using the cDNA as a template and digesting with restriction enzymes.

[0056] In another embodiment of the present method, a DNA sample is first prepared from a subject. Then, a DNA containing a gene encoding a polypeptide of the present
25 invention or its expression control region is amplified. Thereafter, the amplified DNA is dissociated into single strand DNAs, and the single strand DNAs are separated on a non-denaturing gel. The mobility of the separated single strand DNAs on the gel is compared with that of a control.

30 [0057] Such methods include, for example, the PCR-SSCP (single-strand conformation polymorphism) method ("Cloning and polymerase chain reaction-single-strand conformation polymorphism analysis of anonymous Alu repeats on chromosome
11." Genomics 1992, Jan. 1, 12(1): 139-146; "Detection of p53
35 gene mutations in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction

products." Oncogene 1991, Aug. 1, 6(8): 1313-1318). This method is particularly preferable for screening many DNA samples, since it has advantages such as: comparative simplicity of operation; and small amount of a test sample required. The principle of the method is as follows. A single strand DNA dissociated from a double-strand DNA fragment forms a unique higher-order conformation depending on respective nucleotide sequence. Complementary single-stranded DNAs having the same chain length of the dissociated DNA strand shift to different positions in accordance with the difference of the respective higher-order conformations after electrophoresis on a polyacrylamide gel without a denaturant. The higher conformation of a single-stranded DNA changes even by a substitution of one nucleotide, which change results in a different mobility by polyacrylamide gel electrophoresis. Accordingly, the presence of a mutation in a DNA fragment due to point mutation, deletion, insertion and such can be detected by detecting this change in mobility.

[0058] Specifically, DNA containing a gene encoding a polypeptide of the present invention or its expression control region is first amplified by PCR and such. Typically, the range of amplification is preferably about 200 bp to 400 bp in length. Those skilled in the art can appropriately select the condition and such for the PCR. The amplified DNA products can be labeled by using primers labeled with isotopes such as ^{32}P , fluorescent dyes, biotin, and the like during the PCR. Alternatively, the amplified DNA products can also be labeled by conducting PCR in a PCR reaction solution containing substrate nucleotides labeled with isotopes such as ^{32}P , fluorescent dyes, biotin, or the like. Furthermore, the labeling can also be carried out by adding substrate nucleotides labeled with an isotope such as ^{32}P , fluorescent dyes, biotin, or the like to the amplified DNA fragment using Klenow enzyme and such, after the PCR reaction. Then, the obtained labeled DNA fragments are denatured by heating and such, and electrophoresis is carried out on a polyacrylamide

gel without denaturant such as urea. The condition for the separation of the DNA fragments can be improved by adding appropriate amounts (about 5% to 10%) of glycerol to the polyacrylamide gel. Furthermore, although the condition for electrophoresis varies depending on the property of respective DNA fragments, it is usually carried out at room temperature (20°C to 25°C). When a preferable separation can not be achieved at this temperature, a temperature at which optimum mobility can be achieved is searched from temperatures between 4°C to 30°C. The mobility of the DNA fragments is detected by autoradiography with X-ray films, scanner for detecting fluorescence and such, after the electrophoresis to analyze the result. When a band with different mobility is detected, the presence of a mutation can be confirmed by directly excising the band from the gel, amplifying it again by PCR, and directly sequencing the amplified fragment. The bands can also be detected by staining the gel after electrophoresis with ethidium bromide, silver and such, without using labeled DNAs.

[0059] In still another method, a DNA sample is first prepared from a subject. DNA containing a gene encoding a polypeptide of the present invention or its expression control region is amplified, and then, the amplified DNAs are separated on a gel with gradient concentration of a DNA denaturant. The mobilities of the separated DNAs on the gel are compared with those of a control.

[0060] The denaturant gradient gel electrophoresis method (DGGE method) can be exemplified as such methods. The DGGE method comprises the steps of: (1) electrophoresing the mixture of DNA fragments on a polyacrylamide gel with gradient concentration of denaturant; and (2) separating the DNA fragments in accordance with the difference of instabilities of respective fragments. When DNA fragments containing mismatches shift to a part with a certain concentration of the denaturant in the gel, the DNA fragments partly dissociate to single-strand near the mismatches due to the instability of the DNA sequence. The mobility of the partly-dissociated DNA fragment

becomes remarkably slow, giving a difference in mobility compared to that of perfectly double-stranded DNAs without dissociated parts, which allows separation of these DNAs. Specifically, DNA containing a gene encoding a polypeptide of the present invention or its expression control region is (1) amplified by PCR and such with a primer of the present invention and such; (2) electrophoresed on a polyacrylamide gel with gradient concentration of denaturant such as urea; and (3) the result is compared with a control. The presence or absence of a mutation can be detected by detecting the difference of mobility of the DNA fragment due to the extremely slowed down mobility speed of the fragment by separation into single-stranded DNAs of a DNA fragment with mutations at parts of the gel where the concentration of the denaturant is lower.

[0061] In addition to the above-mentioned methods, the Allele Specific Oligonucleotide (ASO) hybridization method can be used to detect mutations only at specific sites. When an oligonucleotide with a nucleotide sequence expected to contain a mutation is prepared and is subjected to hybridization with a DNA sample, the efficiency of hybridization is reduced in case the mutation exists. This can be detected by the Southern blotting method; methods which utilize a particular fluorescent reagent that has a characteristic to quench by intercalation into the gap of the hybrid; and such. Furthermore, the detection may be also conducted by the ribonuclease A mismatch truncation method. Specifically, DNA containing a gene encoding a polypeptide of the present invention is amplified by PCR and such, and the amplified DNAs are hybridized with labeled RNAs, which were prepared from a control cDNA and such that were incorporated into a plasmid vector and such. The presence of a mutation can be detected with autoradiography and such, after cleaving those sites of the hybrid that form a single-stranded conformation due to the existence of a mutation with ribonuclease A.

[0062] Another embodiment of the test method of the present invention includes a method comprising the step of

detecting the expression level of a gene encoding a polypeptide of the present invention. Herein, transcription and translation are included in the phrase "expression of a gene". Accordingly, mRNAs and proteins are included in the phrase
5 "expression product".

[0063] In a preferred embodiment, the present invention provides methods for assaying a cardiac disease which comprises the step of detecting the expression level of the gene encoding the polypeptide of the present invention in a test subject.

10 [0064] The method as described above comprises the steps of: (i) preparing an RNA sample from cells (for example, cardiac muscle cells) of a subject; (ii) measuring the amount of RNA encoding the polypeptide of the present invention in the sample; and (iii) comparing the measured amount of the RNA with
15 a control.

[0065] A Northern blotting method using a probe which hybridizes with the polynucleotide encoding a polypeptide of the present invention; an RT-PCR method using a primer which hybridizes with a polynucleotide encoding the polypeptide of
20 the present invention; and such can be exemplified as such methods.

[0066] Furthermore, a DNA array (Masami Muramatsu and Masashi Yamamoto, New Genetic Engineering Handbook, YODOSHA Co., LTD., 280-284) can also be utilized in the testing for the
25 transcription level of the gene encoding the polypeptide of the present invention. Specifically, first, a cDNA sample prepared from a subject and a basal plate on which polynucleotide probes hybridizing with the polynucleotides encoding the polypeptides of the present invention are fixed are provided. Plural kinds
30 of polynucleotide probes can be fixed on the basal plate in order to detect plural kinds of polynucleotides encoding the polypeptides of the present invention. Preparation of a cDNA sample from a subject can be carried out by methods well known to those skilled in the art. In a preferable embodiment for
35 the preparation of the cDNA sample, first, total RNAs are extracted from a cell of a subject. Examples of cells include

the cardiac muscle cells. The extraction of total RNAs can be carried out, for example, as follows. So long as total RNAs with high purity can be prepared, known methods, kits and such can be used. Then, the cDNA sample is prepared by synthesizing
5 cDNAs with reverse transcriptase using extracted total RNAs as a template. The synthesis of cDNA from total RNAs can be carried out by methods well known in the art. The prepared cDNA sample is labeled for detection according to needs. The labeling substance is not specifically limited so long as it
10 can be detected, and include, for example, fluorescent substances and radioactive elements. The labeling can be carried out by typical methods to those in the art (L. Luo et al., "Gene expression profiles of laser-captured adjacent neuronal subtypes", Nat. Med. 1999, 117-122).

15 [0067] Herein, the term "control" usually refers to the amount of RNA, encoding polypeptides of the present invention, in the RNA sample prepared from cardiac muscle cells of a healthy individual.

[0068] When a significantly altered level of RNA encoding
20 the polypeptide of the present invention compared with control is detected by the aforementioned method, the test subject is judged as being affected with a heart disease associated with abnormal expression of the gene, or is at risk of developing the disease. Moreover, for a test subject who is already known
25 to be affected with a heart disease, the disease is judged as being caused by a change in the expression level of RNA encoding the aforementioned polypeptide.

[0069] Moreover, the test method of the present invention can be conducted as follows by measuring the protein expression
30 level in cells (for example, cardiac muscle cells) of the test subject. First, protein samples are prepared from cells of a test subject. Second, the amount of a polypeptide of the present invention comprised in the protein samples is measured. Third, the measured amount of the polypeptide is compared with
35 control.

[0070] Such methods include SDS polyacrylamide electrophoresis method, and methods using antibodies that bind to a protein of the present invention such as Western blotting method, Dot blotting method, Immunoprecipitation method, Enzyme-linked immunosorbent assay (ELISA) and Immunofluorescence method.

[0071] When significantly altered expression level of a protein encoded by a polynucleotide of the present invention is detected compared with the control, a test subject is judged as being at a risk of developing heart disease in the future (high risk) or already affected with heart disease. Moreover, for a test subject who is already known to be affected with a heart disease, the disease is judged as being caused by the change in the expression level of the polynucleotide as described above.

[0072] [Examples]

Herein below, the present invention will be specifically described using Examples, however, it is not to be construed as being limited thereto.

[0073] [Example 1] Construction of Expression Vectors

Mammalian expression vectors, pCMV-FLAG, pCMV-T7 and pCMV-HA, were designed to express N-terminal FLAG-, T7- and HA-tagged proteins, respectively (G. Takaesu, S. Kishida, A. Hiyama, K. Yamaguchi, H. Shibuya, K. Irie, J. Ninomiya-Tsuji and K. Matsumoto, "TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway." Mol. Cell 2000, 5: 649-658).

[0074] The mammalian expression vector expressing the DIL domain (amino acids (aa) 606-983) of afadin was constructed using pCMV-T7.

[0075] The mammalian expression vectors pCMV-HA-mADIP (aa 1-615), pCMV-HA-rADIP-C (aa 159-613), pCMV-HA-mADIP-C (aa 339-615), pCMV-FLAG-mADIP-C (aa 339-615) and pCMV-FLAG-mADIP-M (aa 152-436), expressing mouse ADIP (mADIP) and rat ADIP (rADIP), were constructed using pCMV-FLAG or pCMV-HA.

[0076] The mammalian expression vector, pCMV-HA- α -actinin-1-C (aa 406-892), expressing α -actinin-1 (human, BC015766; GenBank), was constructed using pCMV-HA.

[0077] Glutathione S-transferase (GST) fusion or maltose-binding protein (MBP) fusion vectors of mADIP and rADIP, MBP-mADIP (aa 1-615), MBP-rADIP-C (aa 159-613), MBP-mADIP-C (aa 339-615), GST-rADIP-C (aa 159-613), GST-mADIP-N (aa 1-226) and GST-mADIP-C (aa 339-615), were constructed using pGEX-KG (K.L. Guan and J.E. Dixon, "Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase." Anal. Biochem. 1991, 192: 262-267) or pMal-C2 (New England Biolabs).

[0078] [Example 2] Preparation of antibody

GST fusion proteins of fragments of rADIP C-terminus (aa 159-613; rADIP), mADIP N-terminus (aa 1-226; mADIP-N) and mADIP C-terminus (aa 339-615; mADIP-C) were produced in *Escherichia coli*, purified and then each of the proteins were used as an antigen to raise rabbit pAb.

[0079] Two pAbs against rADIP-C and mADIP-C, M05 and M01, were used after affinity purification with MBP-rADIP-C (aa 159-613) and MBP-mADIP-C (aa 339-615), respectively. Another pAb against mADIP-N, M57, was used after affinity purification with GST-mADIP-N (aa 1-226).

[0080] GST- and MBP-fusion proteins were purified with glutathione-sepharose beads (Amersham-Pharmacia Biotech) and amylose resin beads (New England Biolabs), respectively.

[0081] A mouse anti-afadin mAb was prepared according to the literature (T. Sakisaka, H. Nakanishi, K. Takahashi, K. Mandai, M. Miyahara, A. Satoh, K. Takaishi, and Y. Takai, "Different behavior of 1-afadin and neurabin-II during the formation and destruction of cell-cell adherens junction." Oncogene 1999, 18: 1609-1617).

[0082] Rat anti-E-cadherin mAb (ECCD2) was a gift from Dr. M. Takeichi (Center for Developmental Biology, RIKEN, Kobe, Japan). Mouse anti-ZO-1 mAb was purchased from Chemicon.

Mouse anti- α -actinin, anti-vinculin and anti-FLAG-M2 mAbs were purchased from Sigma Chemicals. Rabbit anti- α -actin pAb was purchased from Santa Cruz. Mouse anti-T7 mAb was purchased from Novagen.

5 [0083] [Example 3] Cell Culture and protein concentration
HEK293, MDCK, nectin-2 α -L and nectin-2 α - Δ C-L cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum.

10 [0084] Protein concentration was determined using bovine serum albumin as the protein standard (M.M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal. Biochem. 1976, 72: 248-254).

15 [0085] Example 4 Identification of afadin-binding protein
The present inventors performed yeast two-hybrid screening to identify an afadin-binding protein using a region of afadin containing the DIL domain (aa 606-983) as a bait. A schematic structure of afadin is shown in Fig. 1A.

20 [0086] The bait vector, pGBDU-afadin (aa 511-981), was constructed by subcloning an insert encoding the amino acid residues of afadin into pGBDU-C1 (P. James, J. Hallady and E.A. Craig, "Genomic Libraries and a host strain designed for highly efficient two-hybrid selection in yeast." Genetics 1996, 144: 1425-1436).

25 [0087] Yeast two-hybrid libraries constructed from cDNAs derived from 11-days old mouse embryo, rat brain, rat lung and human testis were purchased from Clontech.

30 [0088] Two-hybrid screening using the yeast strain PJ69-4A (MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4 Δ gal80 Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ) was performed as described in the literature (P. James, J. Hallady and E.A. Craig, "Genomic Libraries and a host strain designed for highly efficient two-hybrid selection in yeast." Genetics 1996, 144: 1425-1436). Standard procedures for yeast manipulation were
35 performed as described in the literature (C.A. Kaiser, A. Adams

and D.E. Gottschling, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, N.Y., 1994).

[0089] As a result, the present inventors screened 2×10^5 clones of a mouse embryo library and 2×10^5 clones of a rat brain library and obtained 31 and 25 positive clones, respectively. Four mouse clones and one rat clone encoded proteins similar to the carboxy-terminal portion of human KIAA0923 (AB023140; GenBank/EMBL/DDBJ). The two-hybrid analysis revealed that ADIP specifically binds to the DIL domain of afadin but not to the DIL domain of yeast Myo4 (Fig. 1B). Moreover, yeast transformants with the indicated plasmids were streaked on synthetic complete medium lacking histidine and then incubated for 3 days at 30°C to score HIS3 reporter activity.

[0090] The present inventors named this protein afadin DIL domain-interacting protein (ADIP). ADIP has three coiled-coil domains and the region containing the third coiled-coil domain (aa 339-480) of ADIP was essential for the binding with the DIL domain of afadin (Figs. 2A and 3A).

[0091] [Example 5] Full-length cDNAs of mouse ADIP and rat ADIP

For full-length cDNAs of mouse and rat ADIPs, BLAST searches were conducted against GenBank and EMBL databases. A selection of hits obtained by BLAST searches against the human subset of GenBank and EMBL sequences was used to assemble the cDNA sequence of the human homologue of KIAA0923 (AB023140; GenBank/EMBL/DDBJ). A cDNA of KIAA0923 was provided by Dr. T. Nagase.

[0092] Full-length cDNAs of mADIP (accession number: AF532969) and rADIP (accession number: AF532970) were prepared from mouse and rat brain cDNAs (Clontech), respectively, by reverse-transcriptase-PCR using the following primer sets: for mADIP cDNA, 5'-CGTAGGAGAGTGACAGGAGCTG-3' (SEQ ID NO: 5) and 5'-GGTTATCGAGTTTTTCTACATGAC-3' (SEQ ID NO: 6); and for rADIP cDNA, 5'-CGTAGGAGAGTGACAGGAGCTG-3' (SEQ ID NO: 7) and 5'-TTCCTGTTTTTGCACTGTAGCTG-3' (SEQ ID NO: 8).

[0093] The PCR products were subcloned into pCR4B (Invitrogen) to perform nucleotide sequence analysis according to the dideoxynucleotide termination method using a DNA sequencer (model 3100; Applied Biosystems, Inc.).

5 [0094] The mouse and rat full-length cDNAs encoded proteins composed of 615 aa with a calculated molecular weight of 70,954 Da, and 613 aa with a calculated molecular weight of 70,684 Da, respectively (Fig. 2A).

10 The aa sequences of mouse ADIP (mADIP) and rat ADIP (rADIP) were 92% identical to each other and 88% and 87% identical to that of human KIAA0923, respectively (Fig. 2A).

[0095] [Example 6] Transfection

To confirm whether the isolated cDNAs encode a full-length ADIP, HEK293 cells were transfected with pCMV-HA-mADIP, expressing hemagglutinin (HA)-tagged full-length mADIP. The transfection was conducted using CalPhos mammalian transfection kit (Clontech). Cell extracts from MDCK and HEK293 were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed as described by Laemmli (U.K. Laemmli, 20 "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature 1970, 227: 680-685). Western blotting with three anti-ADIP pAb (M57, M01 and M05; anti-ADIP polyclonal antibodies (pAbs)) was then performed.

25 [0096] As a result, a protein with a molecular weight of approximately 78 kDa, which is similar to the molecular weight of HA-tagged ADIP, was detected in the extracts of HEK293 cells expressing HA-mADIP (Fig. 2B).

[0097] Therefore, the present inventors concluded that the isolated cDNA encodes the full-length ADIP.

30 [0098] [Example 7] Identification of ADIP function:

Binding to α -actinin

To gain insight into the function of ADIP, the present inventors attempted to identify ADIP-binding protein(s). As described above, the ADIP has three coiled-coil domains and the 35 region containing the third coiled-coil domain (aa 339-480) of

the ADIP is required for the binding to the DIL domain of afadin.

[0099] The present inventors performed yeast two-hybrid screening using a region of the ADIP containing three coiled-coil domains in total (aa 152-436) as baits. One bait vector, pGBD-mADIP-B (aa 152-436), was constructed by subcloning an insert encoding the aa residues of mADIP into pGBD-C1 (P. James, J. Hallady and E.A. Craig., "Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast." Genetics 1996, 144: 1425-1436).

[0100] The present inventors screened 7×10^5 clones derived from a rat lung library and obtained 18 positive clones. Nine clones encoded the C terminus of α -actinin-1 (human, BC015766; GenBank). α -Actinin is a well-characterized protein that shows F-actin-crosslinking activity (K. Burridge and J.R. Feramisco, "Non-muscle alpha actinins are calcium-sensitive actin-binding proteins." Nature 1981, 294: 565-567). Four isoforms of human α -actinin have been identified: non-muscle actinin-1 and -4, and muscle actinin-2 and -3 (D.B. Millake, A.D. Blanchard, B. Patel and D.R. Critchley, "The cDNA sequence of a human placental alpha-actinin." Nucleic Acids Res. 1989, 17: 6725; H. Youssoufian, M. McAfee and D.J. Kwiatowski, "Cloning and chromosomal localization of the human cytoskeletal alpha-actinin gene reveals linkage to the beta-spectrin gene." Am. J. Hum. Genet. 1990, 47: 62-71; A.H. Beggs, T.J. Byers, J.H. Knoll, F.M. Boyce, G.A. Bruns and L.M. Kunkel, "Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11." J. Biol. Chem. 1992, 267: 9281-9288; K. Honda, T. Yamada, R. Endo, Y. Ino, M. Gotoh, H. Tsuda, Y. Yamada, H. Chiba and S. Hirohashi, "Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion." J. Cell Biol. 1998, 140: 1383-1393).

[0101] The present analysis suggested that ADIP binds to α -actinin-2 in addition to α -actinin-1 (data not shown), indicating that the binding of ADIP to α -actinin is not specific for α -actinin-1. The analysis also revealed that the

region containing only the first coiled-coil domain (aa 1-226) of ADIP bound to α -actinin-1 (Fig. 3A) and that the two C-terminal EF-hand motifs of α -actinin-1 are necessary for its binding to ADIP (Fig. 9A).

5 [0102] These results indicate that the first coiled-coil domain of ADIP binds to the EF-hand motifs of α -actinin-1.

[0103] [Example 8] Immunoprecipitation and Affinity chromatography

10 To further confirm the binding of ADIP to afadin *in vivo* and *in vitro*, the present inventors performed immunoprecipitation and affinity chromatography analyses.

[0104] Co-immunoprecipitation experiments using HEK293 cells were performed as follows: HEK293 cells were transfected with the expression plasmids in various combinations. The
15 cells were suspended in 1 ml Buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 μ M α -phenylmethanesulfonyl fluoride hydrochloride and 10 μ g/ml aprotinin), sonicated for 10 s for three times at 10 s intervals and then incubated on ice for 30 min. A cell extract
20 (1.2 mg protein) was obtained by centrifugation of the incubated cells at 100,000 x g for 15 min, and the cell extract was pre-cleared by incubation with protein G-Sepharose 4 Fast Flow beads (Amersham-Pharmacia Biotechnology). The cell
25 extract was incubated with 20 μ l of anti-FLAG M2 mAb-coated protein G-Sepharose 4 Fast Flow beads at 4°C for 18 h. The beads were washed with Buffer A, followed by elution of the bound proteins by boiling the beads in SDS sample buffer (60 mM Tris-HCl, pH 6.7, 3% SDS, 2% 2-mercaptoethanol and 5% glycerol) for 10 min. The obtained sample was then subjected to SDS-PAGE,
30 followed by Western blotting.

[0105] Co-immunoprecipitation experiments using MDCK cells were performed as follows: MDCK cells on two 10-cm dishes were sonicated in 2 ml Buffer A, followed by ultracentrifugation at 100,000 x g for 15 min. The cell extract was pre-cleared by
35 incubation with protein A-Sepharose CL-4B beads (Amersham-Pharmacia Biotechnology) and then incubated with 20 μ l of anti-

ADIP pAb (M05)-coated protein A-Sepharose CL-4B beads at 4°C for 18 h. After washing the beads in Buffer A, the bound proteins were eluted by boiling the beads in the SDS sample buffer for 10 min. The samples were then subjected to SDS-PAGE, followed by Western blotting.

[0106] Affinity chromatography using MDCK cells were carried out as follows: MDCK cells on two 10-cm dishes were sonicated in 2 ml buffer, followed by ultracentrifugation at 100,000 x g for 15 min. The supernatant was incubated at 4°C for 18 h with MBP or MBP-mADIP (200 pmol each) immobilized on 20 µl (wet volume) of amylose resin beads (New England Biolabs). After extensively washing the beads in Buffer A, the bound proteins were eluted with Buffer A containing 20 mM maltose. The eluates were boiled in SDS sample buffer. The samples were then subjected to SDS-PAGE, followed by Western blotting with anti-afadin mAb.

[0107] Co-immunoprecipitation of FLAG-tagged mADIP-C with the T7-tagged DIL domain of afadin was conducted. Expression vectors were transfected into HEK293 cells as indicated in Fig. 3B. The T7-tagged DIL domain of afadin specifically co-immunoprecipitated with the FLAG-tagged mADIP-C, as shown by Western blotting with anti-T7 and anti-FLAG mAbs.

[0108] Next, the extract of MDCK cells expressing endogenous afadin was incubated with maltose-binding protein (MBP)-fusion protein of the full-length mADIP (aa 1-615) immobilized on amylose-resin beads. After the beads were washed with lysis buffer, the bound proteins were eluted and the eluate was subjected to SDS-PAGE followed by Western blotting with anti-afadin mAb. Afadin indeed bound to MBP-mADIP, but not to singular MBP (Fig. 3C).

[0109] Finally, to confirm *in vivo* binding of ADIP to afadin, the present inventors examined the extract of MDCK cells whether endogenous afadin co-immunoprecipitates with endogenous ADIP. Endogenous afadin co-immunoprecipitated through the immunoprecipitation of endogenous ADIP in the extract of MDCK cells with anti-ADIP pAb (Fig. 3D). Afadin did

not co-immunoprecipitate with control IgG. Two-hybrid analysis and these results as described above indicate that ADIP binds to afadin both *in vivo* and *in vitro*.

[0110] [Example 10] Tissue and subcellular distribution of
5 ADIP

Northern blotting was performed according to the literature (J. Sambrook, E.F. Fritsch and T. Maniatis, "Molecular Cloning: A Laboratory Manual, 2nd Edition." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., 1989, 10 7.1-7.87). mADIP cDNA fragment (bp 552-3194) was radiolabeled with [α -³²P] by a standard random priming method and was used as a probe in mouse multiple tissue Northern blotting (Clontech).

[0111] As a result, an approximately 4.3-kb mRNA was
15 detected in all mouse tissues, including heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis (Fig. 4A). A smaller band (approximately 3.0 kb) was also detected in the liver and the testis (Fig. 4A, lanes 5 and 8).

[0112] Homogenates of various mouse tissues (30 μ g protein
20 each) were subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting analysis with anti-ADIP pAb (M57). The result detected an approximately 78-kDa protein having the same size as ADIP detected in HEK293 and MDCK cells and in mouse spleen, lung and kidney (Fig. 4B, lanes 3, 4, 7 and 9).
25 Smaller bands (around 60 kDa in heart, around 76 and around 40 kDa in testis) were also detected, suggesting expression of smaller splice variants of ADIP (Fig. 4B, lanes 1 and 8).

[0113] Subcellular fractionation analysis of rat liver was performed as described in the literature (H. Kawabe, H.
30 Nakanishi, M. Asada, A. Fukuhara, K. Morimoto, M. Takeuchi and Y. Takai, "Pilt, a novel peripheral membrane protein at tight junctions in endothelial cells." J. Biol. Chem. 2001, 276: 48350-48355). Each fraction (30 μ g protein each) was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western
35 blotting with anti-ADIP pAb (M57) or anti-afadin mAb. The results showed ADIP to be rich in the fractions of AJs and TJs,

which fraction was also abundant in afadin (Fig. 4C, lanes 4 and 5). The lack of detection of the approximately 78-kDa protein in the liver by the Western blotting analysis of tissue distribution of ADIP (Fig. 4B, lane 5) may be due to its low expression level.

[0114] These results indicate that ADIP is widely expressed, although its expression level varying depending on the tissue type.

[0115] [Example 11] Co-localization of ADIP with afadin at AJs in Epithelial cells

Afadin has been shown to strictly localize at AJs undercoated with F-actin bundles (Mandai et al., 1997). The present inventors examined the co-localization of ADIP with afadin at AJs in MDCK cells by immunofluorescence microscopy.

[0116] The immunofluorescence microscopy of cultured cells and frozen sections of mouse tissues were performed as described in the literatures (K. Mandai, H. Nakanishi, A. Satoh, H. Obaishi, M. Wada, H. Nishioka, M. Itoh, A. Mizoguchi, T. Aoki, T. Fujimoto, Y. Matsuda, S. Tsukita and Y. Takai, "Afadin: A novel actin filament-binding protein with one PDZ domain localized at cadherin-based cell-to-cell adherens junction." J. Cell Biol. 1997, 139: 517-528; K. Takahashi, H. Nakanishi, M. Miyahara, K. Mandai, K. Satoh, A. Satoh, H. Nishioka, J. Aoki, A. Nomoto, A. Mizoguchi and Y. Takai, "Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein." J. Cell Biol. 1999, 145: 539-549). Specifically, MDCK cells were double-stained with various combinations of anti-ADIP (M05), anti-afadin and anti-vinculin Abs.

[0117] The results showed co-localization of ADIP and afadin at cell-cell junctions (Fig. 5A). Substantially the same results were obtained with three anti-ADIP pAbs, M57, M01, and M05.

[0118] Moreover, ADIP was co-stained with Golgi 58 kDa protein, a marker for the Golgi complex. As a result, ADIP in

the perinuclear region was stained at the Golgi complex (Fig. 5A).

[0119] [Example 12] Comparison of ADIP and endogenous afadin localizations

5 The present inventors prepared nectin-2 α -L and nectin-2 α - Δ C-L cell lines stably expressing HA-tagged ADIP (rADIP-C; aa 159-613) using LIPOFECTAMINE 2000 (Invitrogen) to confirm co-localization of ADIP and afadin.

10 [0120] Full-length nectin-2 α (derived from nectin-2 α -L cells) or nectin-2 α lacking 4 amino acids at the C terminus (derived from nectin-2 α - Δ C-L cells) were transiently expressed in cadherin defective L-cells that stably express afadin to compare the localization of expressed proteins with the localization of endogenous afadin. The full-length nectin-2 α
15 was able to bind to afadin, but the nectin-2 α - Δ C failed to bind to afadin (K. Takahashi, H. Nakanishi, M. Miyahara, K. Mandai, K. Satoh, A. Satoh, H. Nishioka, J. Aoki, A. Nomoto, A. Mizoguchi and Y. Takai, "Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens
20 junctions through interaction with Afadin, a PDZ domain-containing protein." J. Cell Biol. 1999, 145: 539-549).

[0121] The result revealed afadin to concentrate at the nectin-2 α -based cell-cell adhesion sites in nectin-2 α -L cells, consistent with the results shown in literatures (M. Miyahara,
25 H. Nakanishi, K. Takahashi, K. Satoh-Horikawa, K. Tachibana and Y. Takai, "Interaction of nectin with afadin is necessary for its clustering at cell-cell contact sites but not for its cis dimerization or trans interaction." J. Biol. Chem. 2000, 275: 613-618; K. Tachibana, H. Nakanishi, K. Mandai, K. Ozaki, W.
30 Ikeda, Y. Yamamoto, A. Nagafuchi, S. Tsukita and Y. Takai, "Two cell adhesion molecules, nectin and cadherin, interact through their cytoplasmic domain-associated proteins." J. Cell Biol. 2000, 150: 1161-1176). Moreover, the endogenously expressed HA-ADIP concentrated at nectin-2 α -based cell-cell adhesion
35 sites and co-localized with afadin in nectin-2 α -L cells (Fig. 6A).

[0122] However, the endogenously expressed HA-ADIP did not concentrated at the cell-cell adhesion site in nectin-2 α - Δ C-L cells (Fig. 6B).

[0123] [Example 13] Precise localization of ADIP in the
5 junctional complex regions

To identify the precise localization of ADIP in the junctional complex regions, a frozen cross-section of mouse small intestine was triple-stained with anti-ADIP pAb, anti-afadin mAb and anti ZO-1 mAb. The frozen cross-section of
10 small intestine was used due to the sharp separation of TJs and AJs in this cell type (M. Itoh, A. Nagafuchi, S. Yonemura, T. Kitani-Yasuda, S. Tsukita and S. Tsukita, "The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in
15 epithelial cells: cDNA cloning and immunoelectron microscopy." J Cell Biol. 1993, 121: 491-502). Furthermore, ZO-1 is known to be a marker for TJs (B.R. Stevenson, J.D. Siliciano, M.S. Mooseker and D.A. Goodenough, "Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction
20 (zonula occludens) in a variety of epithelia." J. Cell Biol. 1986, 103: 755-766; M. Itoh, A. Nagafuchi, S. Yonemura, T. Kitani-Yasuda, S. Tsukita and S. Tsukita, "The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in
25 epithelial cells: cDNA cloning and immunoelectron microscopy." J. Cell Biol. 1993, 121: 491-502).

[0124] Immunoelectron microscopy of mouse small intestine absorptive epithelia cell using ultra-thin frozen section technique was performed according to the literature (K. Mandai,
30 H. Nakanishi, A. Satoh, H. Obaishi, M. Wada, H. Nishioka, M. Itoh, A. Mizoguchi, T. Aoki, T. Fujimoto, Y. Matsuda, S. Tsukita and Y. Takai, "Afadin: A novel actin filament-binding protein with one PDZ domain localized at cadherin-based cell-to-cell adherens junction." J. Cell Biol. 1997, 139:517-528).

[0125] The results showed that ADIP co-localized with afadin, but localized at a slightly more basal side than ZO-1 in the absorptive epithelia (arrows in Figs. 7A and 7B).

[0126] Moreover, it was revealed that ADIP exclusively
5 localized at AJs undercoated with F-actin bundles and did not
localize at TJs and desmosomes (Fig. 7C). This localization
pattern of ADIP was identical to that of afadin and nectin but
was different from that of E-cadherin, which is concentrated at
10 AJs but is more widely distributed from the apical to basal
sides of the lateral plasma membranes (K. Mandai, H. Nakanishi,
A. Satoh, H. Obaishi, M. Wada, H. Nishioka, M. Itoh, A.
Mizoguchi, T. Aoki, T. Fujimoto, Y. Matsuda, S. Tsukita and Y.
Takai, "Afadin: A novel actin filament-binding protein with one
PDZ domain localized at cadherin-based cell-to-cell adherens
15 junction." J. Cell Biol. 1997, 139:517-528; K. Takahashi, H.
Nakanishi, M. Miyahara, K. Mandai, K. Satoh, A. Satoh, H.
Nishioka, J. Aoki, A. Nomoto, A. Mizoguchi and Y. Takai,
"Nectin/PRR: an immunoglobulin-like cell adhesion molecule
recruited to cadherin-based adherens junctions through
20 interaction with Afadin, a PDZ domain-containing protein." J.
Cell Biol. 1999, 145: 539-549).

[0127] These results indicated that ADIP co-localize with afadin and nectin at cell-cell AJs undercoated with F-actin bundles.

25 [0128] [Example 14] ADIP signal

The ADIP signal was not detected at focal adhesion sites where the vinculin signal was detected in MDCK cells (Fig. 5B).

[0129] In the heart, a focal adhesion site, named costamere, is well developed and periodically located along the
30 lateral borders of cardiac muscle cells (L. Terracio, D.G. Simpson, L. Hilenski, W. Carver, R.S. Decker, N. Vinson and T.K. Borg, "Distribution of vinculin in the Z-disk of striated muscle: analysis by laser scanning confocal microscopy." J. Cell Physiol. 1990, 145: 78-87). Vinculin, but not nectin and
35 afadin, is known to be localized at costameres (K. Mandai, H. Nakanishi, A. Satoh, H. Obaishi, M. Wada, H. Nishioka, M. Itoh,

A. Mizoguchi, T. Aoki, T. Fujimoto, Y. Matsuda, S. Tsukita and Y. Takai, "Afadin: A novel actin filament-binding protein with one PDZ domain localized at cadherin-based cell-to-cell adherens junction." J. Cell Biol. 1997, 139: 517-528; K. Takahashi, H. Nakanishi, M. Miyahara, K. Mandai, K. Satoh, A. Satoh, H. Nishioka, J. Aoki, A. Nomoto, A. Mizoguchi and Y. Takai, "Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein." J. Cell Biol. 1999, 145: 539-549).

[0130] The ADIP signal was not detected at costameres where the vinculin signal was detected (Fig. 7D, arrows).

[0131] Both the ADIP and vinculin signals were detected at the intercalated disc, corresponding to the cell-cell AJs (Fig. 7D, arrowheads).

[0132] These results indicate that ADIP does not localize at the cell-matrix junctions.

[0133] [Example 15] Ca^{2+} switch assay

Next, the present inventors investigated the localization of ADIP in comparison with the localization of afadin during destruction and formation of AJs in MDCK cells.

[0134] A Ca^{2+} switch assay using MDCK cells was performed as described in the literature (J. Kartenbeck, M. Schmelz, W.W. Franke and B. Geiger, "Endocytosis of junctional cadherins in bovine kidney epithelial (MDBK) cells cultured in low Ca^{2+} ion medium." J. Cell Biol. 1991, 113: 881-892). Briefly, MDCK cells (1×10^5) were seeded on an 18-mm glass coverslip in a 12-well culture dish. After 48 hrs, the cells were washed with phosphate buffered saline (PBS) and cultured at 2 mM Ca^{2+} in serum-free DMEM for 60 min. Then, cells were cultured at 2 μM Ca^{2+} (in DMEM containing 5 mM EGTA) for 120 min. After the incubation, cells were washed with PBS and cultured again at 2 mM Ca^{2+} in serum-free DMEM for 60 min. When cells were treated with phorbol ester, i.e., 12-o-tetradecanoylphorbol-13-acetate (TPA), the cells were washed with PBS and cultured at 2 mM Ca^{2+} in serum-free DMEM for 60 min. The cells were then cultured at

2 μM Ca^{2+} with 5 mM EGTA for 120 min. After the incubation, 100 nM TPA was added to the medium and then the cells were cultured for another 60 min.

[0135] As a result, due to the switching of the Ca^{2+} concentration in the culture media from 2 mM to 2 μM , MDCK cells gradually dissociated from each other as described in the literature (J. Kartenbeck, M. Schmelz, W.W. Franke and B. Geiger, "Endocytosis of junctional cadherins in bovine kidney epithelial (MDBK) cells cultured in low Ca^{2+} ion medium." J. Cell Biol. 1991, 113:881-892). Afadin, E-cadherin and ADIP signals were highly concentrated at the cell-cell adhesion sites in cells cultured at 2 mM Ca^{2+} (Fig. 8A). The E-cadherin signal disappeared and partly emerged on intracellular vesicles upon cultivation of these cells at 2 μM Ca^{2+} for 120 min (E-cadherin shown in Fig. 8B). Most of the afadin signal remained at the free surface of the cytoplasmic membrane and formed a ring-like structure (afadin in Fig. 8B) as described in the literatures (T. Sakisaka, H. Nakanishi, K. Takahashi, K. Mandai, M. Miyahara, A. Satoh, K. Takahashi and Y. Takai, "Different behavior of 1-afadin and neurabin-II during the formation and destruction of cell-cell adherens junction." Oncogene 1999, 18: 1609-1617; A. Fukuhara, K. Irie, H. Nakanishi, K. Takekuni, T. Kawakatsu, W. Ikeda, A. Yamada, T. Katata, T. Honda, T. Sato, K. Shimizu, H. Ozaki, H. Horiuchi, T. Kita and Y. Takai, "Involvement of Nectin in the Localization of Junctional Adhesion Molecule at Tight Junctions." Oncogene. In press.). The majority of the ADIP signal disappeared from the free surface of the cytoplasmic membrane under the conditions described above (ADIP in Fig. 8B).

[0136] Next, MDCK cells pre-cultured at 2 μM Ca^{2+} for 120 min were cultured again at 2 mM Ca^{2+} for 60 min to examine the accumulation of ADIP at the cell-cell adhesion sites in comparison with E-cadherin accumulation. After the incubation at 2 mM Ca^{2+} , the E-cadherin signal concentrated again at the cell-cell adhesion sites where afadin localized (E-cadherin in

Fig. 8C). The ADIP signal also concentrated again at the cell-cell adhesion sites (ADIP in Fig. 8C).

[0137] Furthermore, the present inventors revealed that a TJ-like structure is formed and afadin and ZO-1, but not E-cadherin, accumulate at this structure, upon the cultivation of MDCK cells at 2 μM Ca^{2+} for 120 min prior to the incubation at 2 μM Ca^{2+} with TPA for 60 min (M.S. Balda, L. Gonzalez-Mariscal, K. Matter, M. Cereijido and J.M. Anderson, "Assembly of the tight junction: the role of diacylglycerol." J. Cell Biol. 1993, 123: 293-302; T. Asakura, H. Nakanishi, T. Sakisaka, K. Takahashi, K. Mandai, M. Nishimura, T. Sasaki and Y. Takai, "Similar and differential behaviour between the nectin-afadin-ponsin and cadherin-catenin systems during the formation and disruption of the polarized junctional alignment in epithelial cells." Genes Cells 1999, 4: 573-581; A. Fukuhara, K. Irie, H. Nakanishi, K. Takekuni, T. Kawakatsu, W. Ikeda, A. Yamada, T. Katata, T. Honda, T. Sato, K. Shimizu, H. Ozaki, H. Horiuchi, T. Kita and Y. Takai, "Involvement of Nectin in the Localization of Junctional Adhesion Molecule at Tight Junctions." Oncogene. In Press; A. Fukuhara, K. Irie, A. Yamada, T. Katata, T. Honda, K. Shimizu, H. Nakanishi and Y. Takai, "Role of Nectin in Organization of Tight Junctions in Epithelial Cells." Genes Cells. In press.). Moreover, afadin signal localized at the TPA induced TJ-like structure. However, ADIP and E-cadherin signals did not localize at the structure (Fig. 8D).

[0138] These results indicate that ADIP shows a different localization from that of afadin during the destruction and formation of the junctional complex.

[0139] [Example 16] *In vivo* and *in vitro* binding of ADIP to α -actinin-1

The present inventors performed immunoprecipitation analysis to confirm the *in vivo* and *in vitro* binding of ADIP to α -actinin-1.

[0140] HEK293 cells were co-transfected with HA-tagged C terminus of α -actinin-1 (HA- α -actinin-1-C; aa 406-892) and FLAG-tagged ADIP (FLAG-mADIP-M; aa 152-436).

[0141] When FLAG-tagged ADIP was immunoprecipitated with anti-FLAG mAb from the cell extract, co-immunoprecipitation of HA- α -actinin-1-C was revealed by Western blotting with HA mAb (Fig. 9B).

5 [0142] Endogenous α -actinin co-immunoprecipitated (Fig. 9C) when FLAG-mADIP-M was immunoprecipitated with anti-FLAG Ab from the extract of HEK293 cell transiently expressing FLAG-tagged mADIP (FLAG-mADIP-M; aa 152-436) alone.

10 [0143] Finally, endogenous α -actinin co-immunoprecipitated (Fig. 9D) when endogenous ADIP was immunoprecipitated with anti-ADIP pAb from MDCK cell extracts. α -Actinin did not co-immunoprecipitate with control IgG.

[0144] These results indicated that ADIP binds to α -actinin both *in vivo* and *in vitro*.

15 [0145] [Example 17] Co-localization of ADIP with α -actinin at AJs in MDCK cells

α -actinin has been demonstrated to interact with α -catenin and localize at AJs (K.A. Knudsen, A.P. Soler, K.R. Johnson and M.J. Wheelock, "Interaction of alpha-actinin with the cadherin/catenin cell-cell adhesion complex via alpha-catenin." J. Cell Biol. 1995, 130: 67-77). Therefore, the present inventors examined whether ADIP co-localizes with α -actinin at AJs in MDCK cells by immunofluorescence microscopy.

20 [0146] The result showed co-localization of ADIP and α -actinin at the cell-cell junctions (Fig. 10A). Moreover, α -actinin but not ADIP localized at limited adherens sites (data not shown).

30 [0147] The frozen sections of mouse small intestine were double-stained with anti-ADIP pAb and anti- α -actinin mAb in order to confirm co-localization of ADIP and α -actinin at the junctional complex regions.

[0148] The result showed that ADIP co-localizes with α -actinin at the absorptive epithelia (Fig. 10B). The localization of ADIP was strictly restricted to the AJs as
35 described above. However, α -actinin was widely distributed

from the apical to basal sides of the lateral plasma membranes (Fig. 10B).

[0149] These results indicate that ADIP forms a complex with afadin and α -actinin at the cell-cell AJs undercoated with F-actin bundles.

[0150] [Effects of the Invention]

The present inventors have provided a novel afadin DIL domain binding protein (ADIP) and a gene encoding the protein. The protein of the present invention is expected to be useful for evaluating actin cytoskeleton-controlling agents.

[0151] Moreover, the protein of the present invention is useful as a functional marker of the intercalate disc for heart diseases, such as myocardial infarction and myocarditis, due to its extremely high expression at the intercalate disc of cardiac muscle cells. Diagnosis of cardiac diseases can be accomplished using the expression level of the gene of the present invention as an index. Moreover, the protein of the present invention is useful for screening for candidate compounds for agents to treat cardiac diseases.

[0152] [Sequence Listing]

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| | | | | | |
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〈213〉 *Rattus norvegicus*

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35

80

85

90

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[Brief Description of the Drawings]

25 [Fig. 1] A diagram and photograph depicting the binding of
 ADIP to the DIL domain of afadin are shown.

(A): schematic illustration of the structure of afadin. RA, Ras-associated domain; FHA, Forkhead-associated domain; DIL, Dilute domain; PDZ, PDZ domain; and PR, proline-rich domain.

30 (B): results of yeast two-hybrid assay, indicating the specific
 binding of ADIP to the DIL domain of afadin.

[Fig. 2] A diagram and photograph depicting the amino acid sequences of ADIP are shown.

(A): deduced amino acid sequences of rat ADIP, mouse ADIP and human KIAA0923. Identical sequences are highlighted in gray.

35 Putative coiled-coil domains are underlined.

(B): result of comparison of molecular weight of native and recombinant ADIP proteins. pCMV HA mADIP was transfected into HEK293 cells and the cell extract was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-ADIP pAb (M57). The extracts derived from control HEK293 and MDCK cells were similarly subjected to SDS-PAGE, followed by Western blotting. Lane 1, control HEK293 cells (1 μ g protein); lane 2, pCMV HA mADIP-transfected HEK293 cells (1 μ g protein); and lane 3, MDCK cells (1 μ g of protein).

[Fig. 3] A diagram and photographs depicting the *in vitro* and *in vivo* binding of ADIP to afadin are shown.

(A): schematic illustration of the structure of mouse ADIP. CC, coiled-coil domain. Yeast two-hybrid analysis of the afadin- or α -actinin-binding regions of ADIP. ADIP, afadin (DIL domain) and α -actinin were constructed into pGBDU or pGAD and co-transfected into reporter yeast strains. As shown by the expression of the HIS3 and ADE2 reporter genes, the binding of ADIP to afadin or α -actinin was determined by evaluating growth of the yeast strains on synthetic complete media lacking histidine and adenine, respectively. +, with interaction; -, no interaction; NT, not tested.

(B): co-immunoprecipitation of the T7-tagged DIL domain of afadin with the FLAG-tagged mADIP-C. Expression vectors were transfected into HEK293 cells as indicated. The T7-tagged DIL domain of afadin specifically co-immunoprecipitated with the FLAG-tagged mADIP-C, as shown by Western blotting with the anti-T7 and anti-FLAG mAbs.

(C): *in vitro* binding of afadin to MBP-mADIP. The extract of MDCK cells was incubated with either MBP or MBP-mADIP (full-length) immobilized on amylose resin beads. The beads were then subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with anti-afadin mAb.

(D): co-immunoprecipitation of endogenous afadin with endogenous ADIP from MDCK cells. The extracts of MDCK cells were immunoprecipitated with the anti-ADIP antibody (M05) and analyzed by Western blotting with anti-ADIP pAb (M05) and anti-

afadin mAb. The results represent three independent experiments.

[Fig. 4] Photographs depicting tissue and subcellular distribution of ADIP are shown.

5 (A): Northern blotting analysis. Mouse RNA blotted membranes (Clontech) were hybridized with the ^{32}P -labeled fragment (bp 552-3194) of the ADIP cDNA, followed by autoradiography. Lane 1, heart; Lane 2, brain; Lane 3, spleen; Lane 4, lung; Lane 5, liver; Lane 6, skeletal muscle; Lane 7, kidney; and Lane 8, testis (short exposure).
10

(B): Western blotting analysis. The homogenates of various mouse tissues (30 μg protein each) were subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with anti-ADIP Ab (M57). Lane 1, heart; Lane 2, brain; Lane 3, spleen; Lane 4, lung; Lane 5, liver; Lane 6, skeletal muscle; Lane 7, kidney; Lane 8, testis; and Lane 9, MDCK cells.
15

(C): subcellular distribution of ADIP in rat liver. Subcellular fractionation of rat liver was performed, and each fraction (30 μg protein each) was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with anti-ADIP pAb (M57) or the anti-afadin mAb. Lane 1, homogenate fraction; Lane 2, soluble fraction; Lane 3, pellet fraction; Lane 4, fraction rich in bile ducts; Lane 5, fraction rich in AJs and TJs. The results represent three independent experiments.
20
25

[Fig. 5] Photographs of the localization of ADIP at AJs in MDCK cells are shown. MDCK cells were double-stained with various combinations of anti-ADIP (M05), anti-afadin and anti-vinculin Abs.

30 (A): ADIP and afadin.

(B): ADIP and vinculin. Bars, 10 μm . The results represent three independent experiments.

[Fig. 6] Photographs of the co-localization of ADIP with afadin in nectin-2 α -L cells are shown.

35 (A): supplementation of ADIP to the nectin-2 α -based cell-cell adhesion sites. Nectin-2a-L cells transiently expressing HA-

tagged ADIP (rADIP-C; aa 159-613) were double-stained with anti-HA and anti-afadin mAb. Arrows show nectin-2 α -based cell-cell adhesion sites.

(B): lack of supplementation of ADIP to the nectin-2 α - Δ C-based cell-cell adhesion sites. Nectin-2 α - Δ C-L cells transiently expressing HA-tagged ADIP (rADIP-C; aa 159-613) were double-stained with anti-HA and anti-afadin mAb. Arrows show nectin-2 α - Δ C-based cell-cell adhesion sites. Bars, 10 μ m. The results represent three independent experiments.

[Fig. 7] Photographs of the co-localization of ADIP and afadin at AJs in mouse small intestine absorptive epithelial cells are shown.

(A) and (B): localization of ADIP, afadin and ZO-1. In (A), the frozen sections were triple-stained with anti-ADIP pAb (M01), anti-afadin and anti-ZO-1 mAb. In (B), the frozen sections were double-stained with anti-ADIP pAb (M01) and anti-afadin mAb (Left panels) or with anti-ADIP pAb (M01) and anti-ZO-1 mAb (right panels). Arrowheads, ADIP signals co-localized with the afadin signal; arrows, ADIP signals localized at a slightly more basal side than the ZO-1 signal; and bars, 10 μ m.

(C): ultrastructural localization of ADIP in mouse small intestine absorptive epithelial cells. The mouse small intestine absorptive epithelial cells were labeled with anti-ADIP pAb (M01) using the ultra-thin frozen section technique. AJ, adherens junctions; DS, desmosome; TJ, tight junctions; and bars, 0.1 μ m.

(D): absence of ADIP at cell-matrix junctions in mouse cardiac cells. The frozen sections were double-stained with anti-ADIP pAb (M01) and anti-vinculin mAb. Arrowheads, intercalated disc; arrows, costamere; and bars, 50 μ m. The results represent three independent experiments.

[Fig. 8] Photographs depicting the behavior of ADIP during destruction and formation of junctional complex consisting of AJs and TJs in MDCK cells are shown. MDCK cells were triple-stained with anti-ADIP (M05), anti-afadin and anti-E-cadherin Ab.

(A): localization of ADIP, afadin and E-cadherin in cells cultured at 2 mM Ca^{2+} (normal Ca^{2+}).

(B): disappearance of immunofluorescence signal of ADIP in the plasma membrane due to the change of Ca^{2+} concentration from normal to low in the media. MDCK cells were incubated at 2 μM Ca^{2+} for 120 min (low Ca^{2+}).

(C): supplementation of ADIP into the cell-cell adhesion sites due to the change of Ca^{2+} concentration from low to normal in the media. MDCK cells were cultured at 2 μM Ca^{2+} for 120 min and then incubated at 2 mM Ca^{2+} for 60 min (low Ca^{2+} + normal Ca^{2+}).

(D): lack of supplementation of ADIP at TPA-induced TJ-like structure. MDCK cells were cultured at 2 μM Ca^{2+} for 120 min and then incubated at 100 nM TPA for 60 min (low Ca^{2+} + TPA).

Bars, 10 μm . The results represent three independent experiments.

[Fig. 9] Photographs depicting the *in vivo* binding of ADIP to α -actinin are shown.

(A): schematic illustration of the structure of α -actinin-1. CH, carponin homologous domain; SPEC, spectrin-like repeat; and EF, EF hand. Yeast two-hybrid analysis of the ADIP-binding regions of α -actinin-1. As indicated by the expression of the HIS3 and ADE2 reporter genes, the binding of α -actinin-1 to ADIP was determined by evaluating the growth of the yeast strain on synthetic complete medium lacking histidine and adenine, respectively. +, interacted; +/-, weakly interacted; and -, not interacted.

(B): co-immunoprecipitation of the HA-tagged α -actinin-1-C with the FLAG-tagged mADIP-M. Expression vectors were transfected into HEK293 cells as indicated. The HA-tagged α -actinin-1-C specifically co-immunoprecipitated with the FLAG-tagged mADIP-M, as shown by Western blotting with anti-HA and anti-FLAG mAbs.

(C): co-immunoprecipitation of endogenous α -actinin with the FLAG-tagged mADIP-M. Expression vectors were transfected into HEK293 cells as indicated. Endogenous α -actinin-1 specifically

co-immunoprecipitated with FLAG-tagged mADIP-M, as shown by Western blotting with anti- α -actinin pAb and anti-FLAG mAb.

(D): co-immunoprecipitation of endogenous α -actinin and endogenous afadin with endogenous ADIP from MDCK cells. The
5 extracts of MDCK cells were immunoprecipitated with anti-ADIP antibody (M05) and analyzed by Western blotting with anti-ADIP (M05), anti- α -actinin and anti-afadin Abs. The results represent three independent experiments.

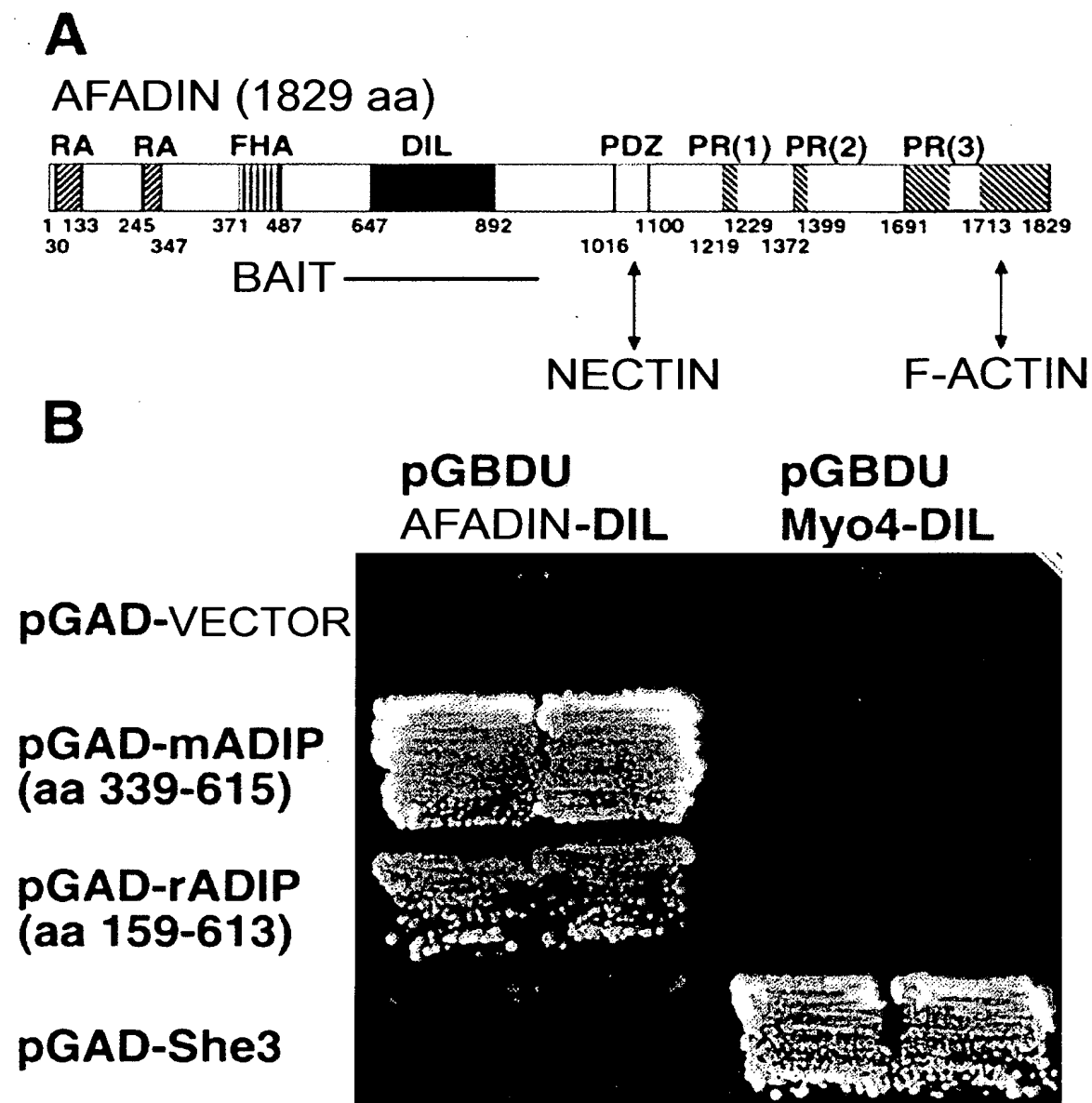
[Fig. 10] Photographs depicting the localization of α -actinin and ADIP at AJs in MDCK cells and mouse small intestine
10 absorptive epithelial cells are shown.

(A): localization of ADIP and α -actinin in MDCK cells. Cells were double-stained with anti-ADIP pAb (M05) and anti- α -actin mAb.

15 (B): localization of ADIP and α -actinin in mouse small intestine absorptive epithelial cells. The frozen sections were tripple-stained with anti-ADIP pAb (M01) and anti- α -actin mAb. Bars, 10 μ m. The results represent three independent experiments.

[Document Name] Drawings

[Fig. 1]

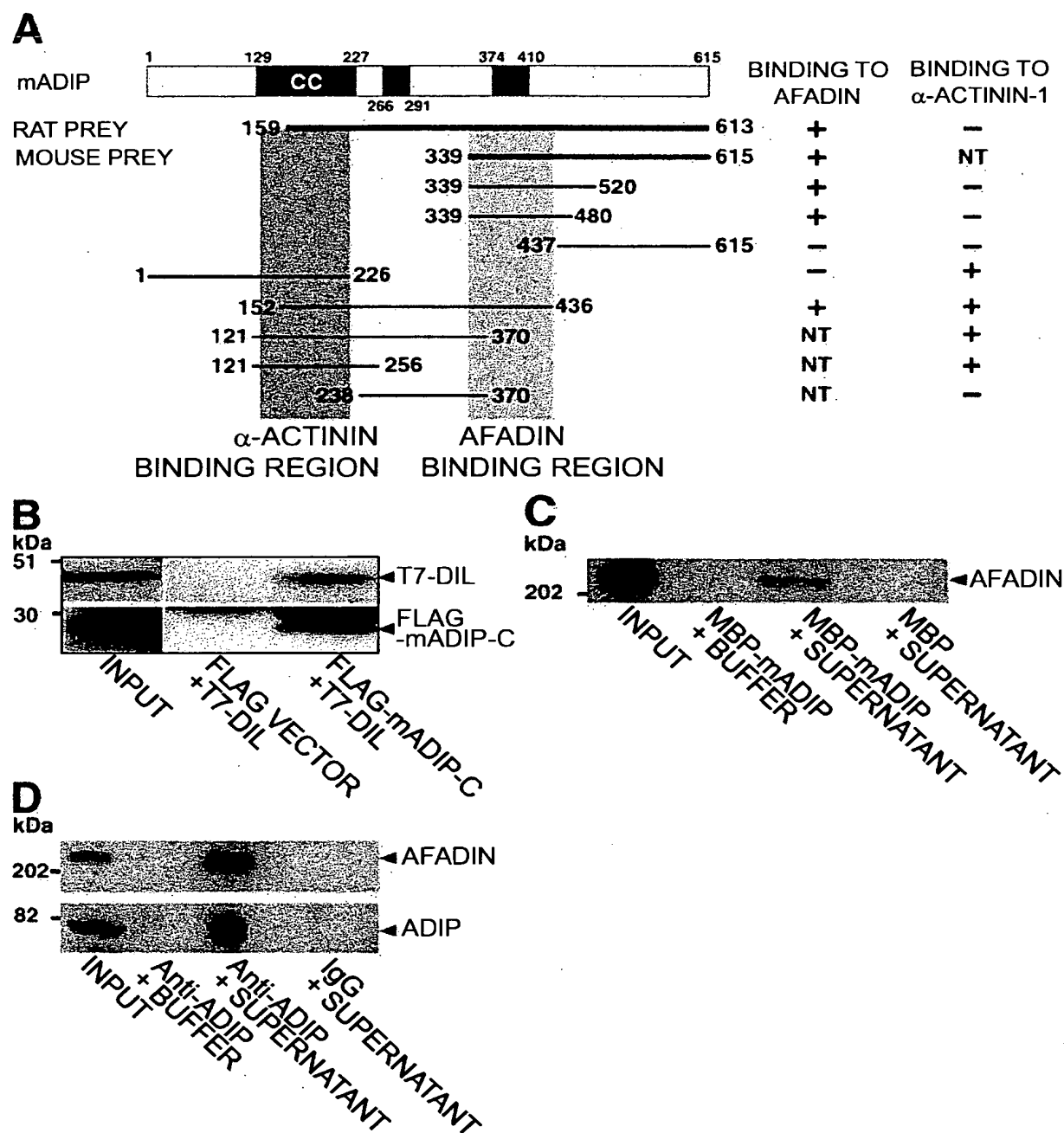


A

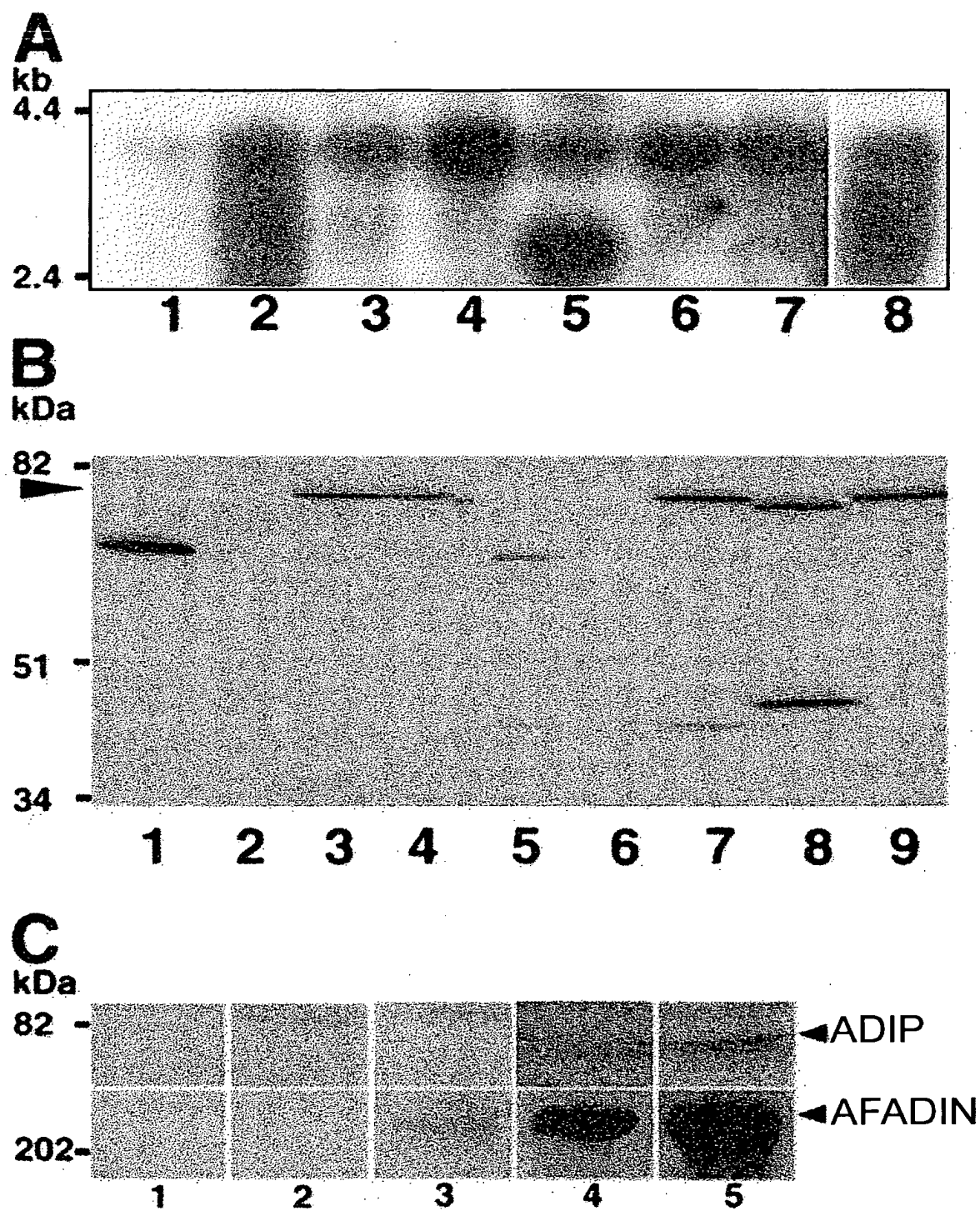
B

HEK293 HEK293 MDCK
HA-mADIP

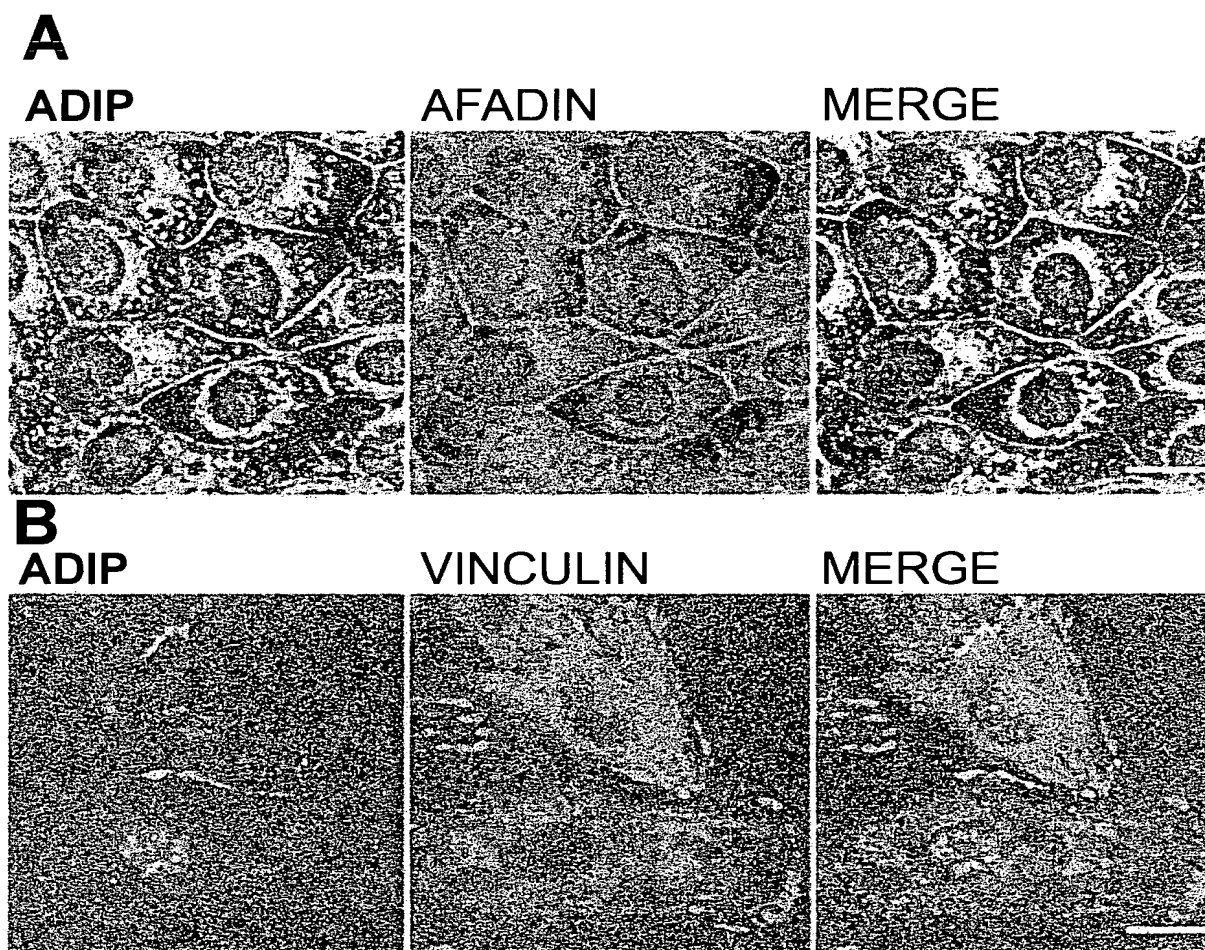
[Fig. 3]



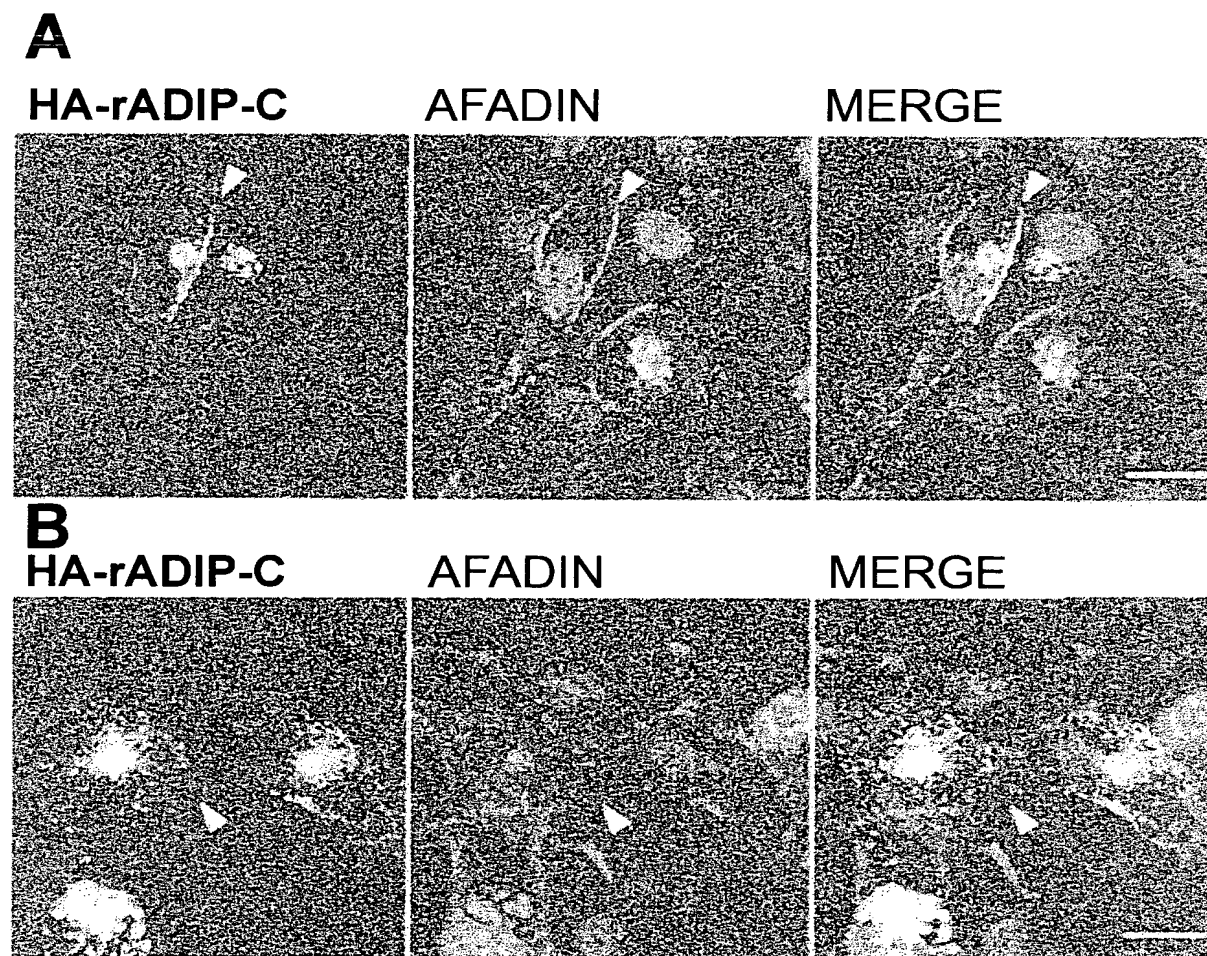
[Fig. 4]



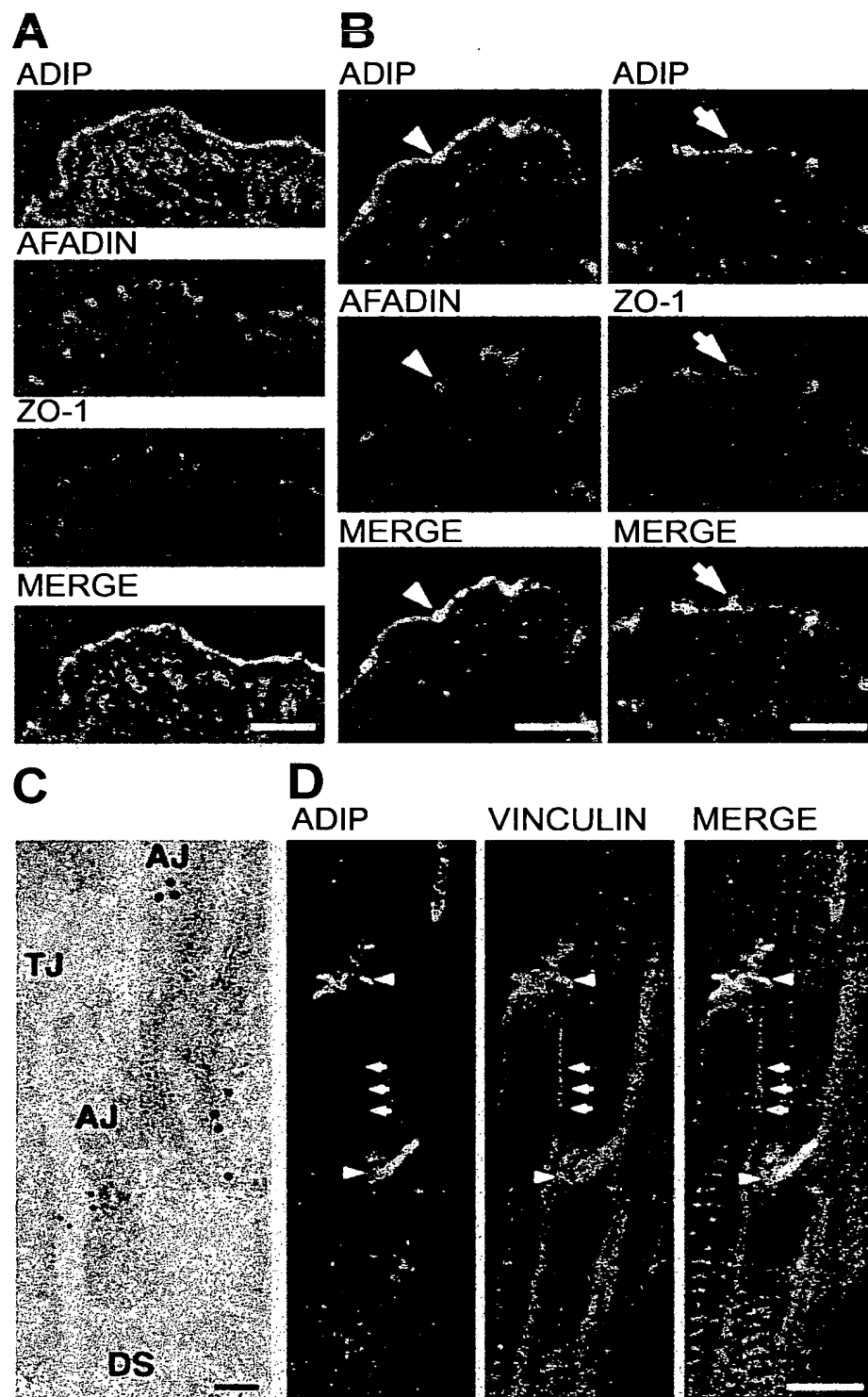
[Fig. 5]



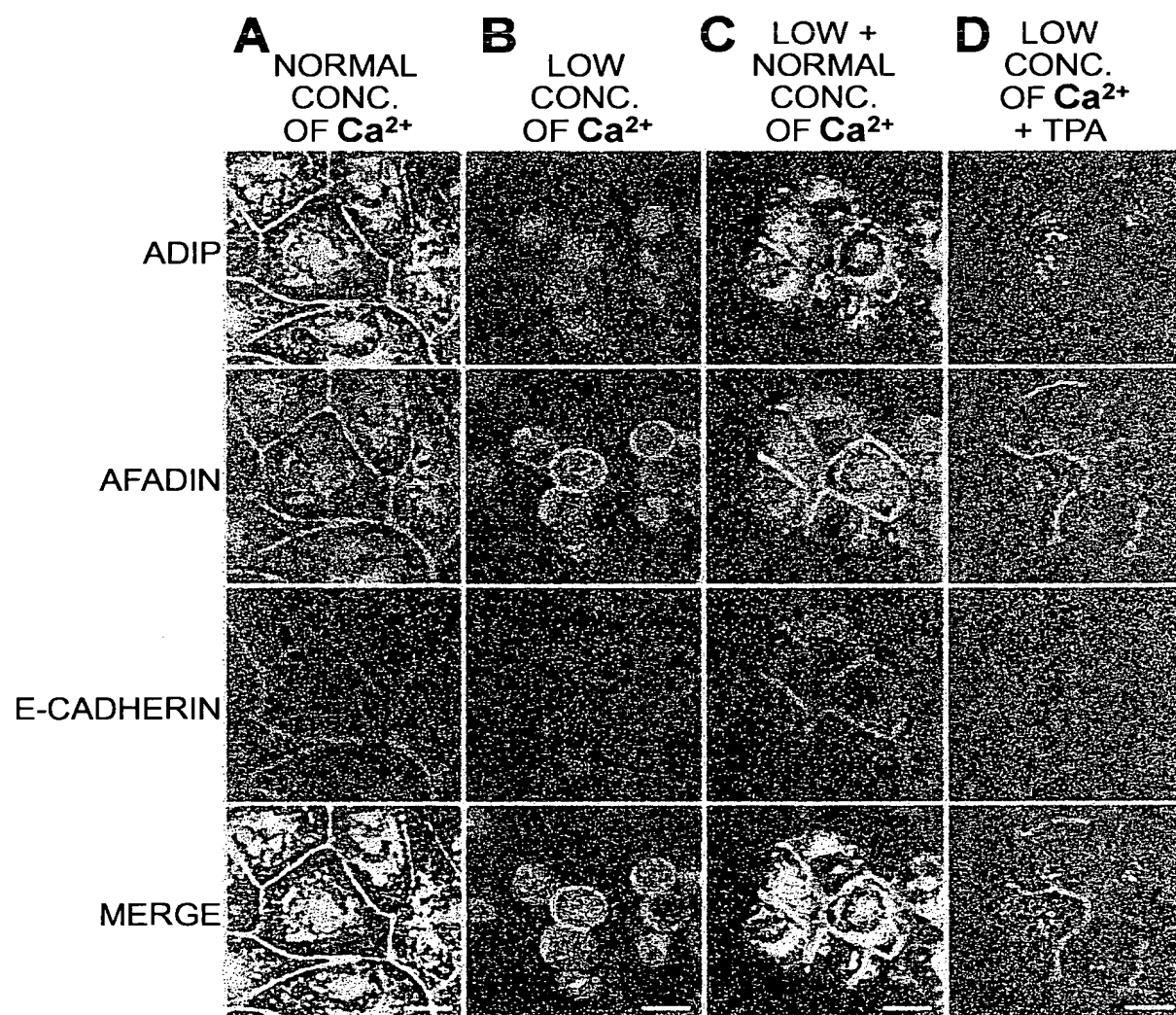
[Fig. 6]



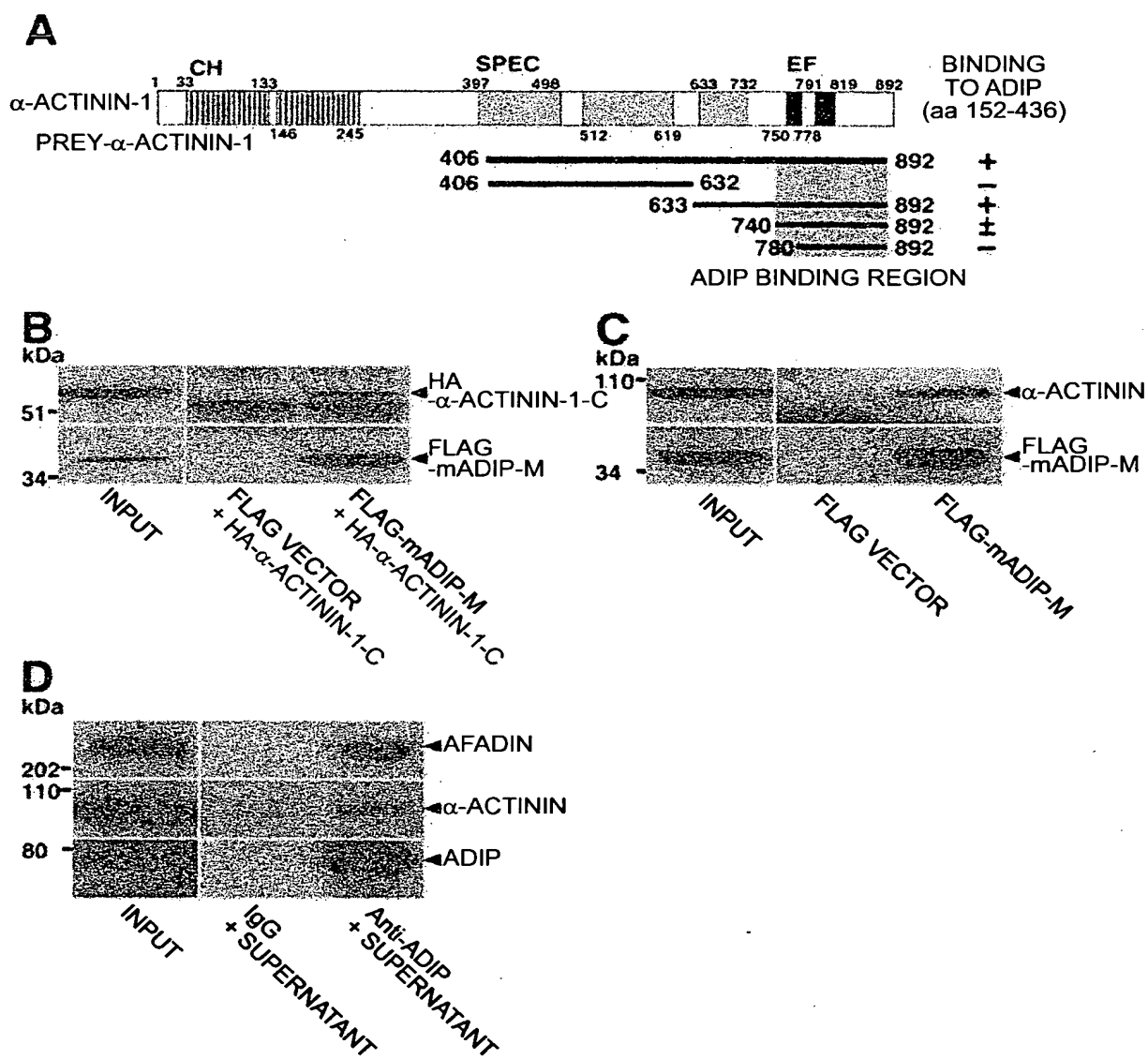
[Fig. 7]



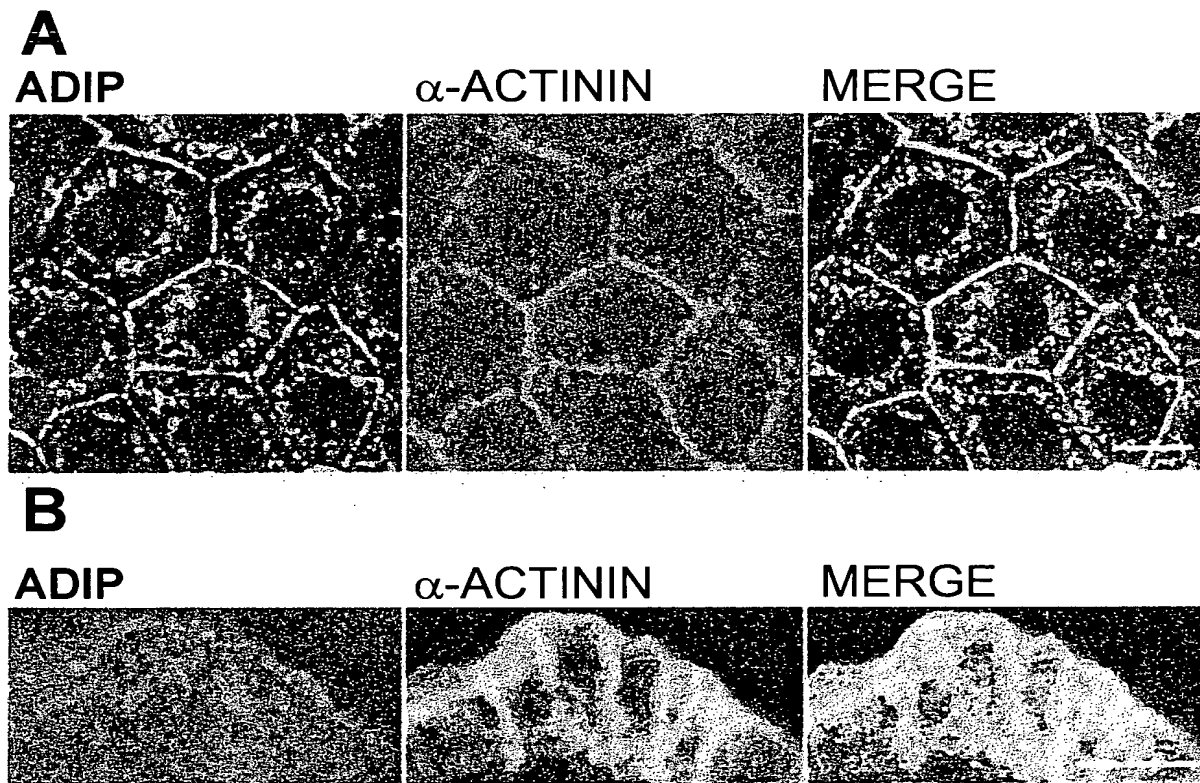
[Fig. 8]



[Fig. 9]



[Fig. 10]



[Document Name] Abstract

[Abstract]

[Problems to be Solved] An objective of the present invention is to provide a gene encoding novel afadin DIL domain-binding protein (ADIP) and use of the novel ADIP protein.

5 [Means to Solve the Problems] The present inventors successfully identified a novel afadin-binding protein (ADIP) using yeast two-hybrid screening in order to identify how the nectin and afadin system organize tight junctions and adherens
10 junctions at the cell-cell junctions. The novel protein is useful for evaluating actin cytoskeleton-controlling agents.

[Selected Drawings] None